

# HAMILTON, BROOK, SMITH & REYNOLDS, P.C.

<b>UTILITY PATENT APPLICATION TRANSMITTAL</b> <small>(Only for new nonprovisional applications under 37 C.F.R. 1.53(b))</small>	Attorney Docket No.	0050.1618000
	First Named Inventor or Application Identifier	Leonard Guarente
	Express Mail Label No.	EL192648508US

Title of Invention	METHODS FOR IDENTIFYING AGENTS WHICH ALTER HISTONE PROTEIN ACETYLATION, DECREASE AGING, INCREASE LIFESPAN
-----------------------	--

<b>APPLICATION ELEMENTS</b> See MPEP chapter 600 concerning utility patent application contents.	ADDRESS TO: Assistant Commissioner for Patents Box Patent Application Washington, D.C. 20231
---	--

1. <input type="checkbox"/> Fee Transmittal Form <i>(Submit an original, and a duplicate for fee processing)</i>  2. <input checked="" type="checkbox"/> Specification <b>Total Pages [98]</b> <i>(preferred arrangement set forth below)</i> - Descriptive title of the invention - Cross References to Related Applications - Statement Regarding Fed sponsored R & D - Reference to microfiche Appendix - Background of the Invention - Summary of the Invention - Brief Description of the Drawings - Detailed Description - Claim(s) - Abstract of the Disclosure  3. <input checked="" type="checkbox"/> Drawing(s) (35 U.S.C. 113) <b>Total Sheets [ 27 ]</b> <input type="checkbox"/> Formal <input checked="" type="checkbox"/> Informal  4. <input type="checkbox"/> Oath or Declaration/POA <b>[Total Pages [ ] ]</b> a. <input type="checkbox"/> Newly executed (original or copy)  b. <input type="checkbox"/> Copy from a prior application (37 C.F.R. 1.63(d)) <i>(for continuation/divisional with Box 17 completed)</i> <b>[NOTE Box 5 below]</b> i. <input type="checkbox"/> <b>DELETION OF INVENTOR(S)</b> Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. 1.63(d)(2) and 1.33(b).  5. <input type="checkbox"/> Incorporation By Reference <i>(useable if Box 4b is checked)</i> The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.	6. <input type="checkbox"/> Microfiche Computer Program <i>(Appendix)</i>  7. <input type="checkbox"/> Nucleotide and/or Amino Acid Sequence Submission <i>(if applicable, all necessary)</i> a. <input type="checkbox"/> Computer Readable Copy  b. <input type="checkbox"/> Paper Copy (identical to computer copy) <b>[ ] Pages</b>  c. <input type="checkbox"/> Statement verifying identity of above copies  <div style="border: 1px solid black; padding: 5px; margin-top: 10px;"> <b>ACCOMPANYING APPLICATION PARTS</b>          8. <input type="checkbox"/> Assignment Papers (cover sheet &amp; documents)          9. <input type="checkbox"/> 37 C.F.R. 3.73(b) Statement <input type="checkbox"/> Power of Attorney  <i>(when there is an assignee)</i>          10. <input type="checkbox"/> English Translation Document <i>(if applicable)</i>          11. <input type="checkbox"/> Information Disclosure Statement (IDS)/PTO-1449 <input type="checkbox"/> Copies of IDS Citations          12. <input type="checkbox"/> Preliminary Amendment          13. <input checked="" type="checkbox"/> Return Receipt Postcard (MPEP 503) (2)  <i>(Should be specifically itemized)</i>          14. <input type="checkbox"/> Small Entity Statement <input type="checkbox"/> Statement filed in prior application,          Statement(s) status still proper and desired          15. <input type="checkbox"/> Certified Copy of Priority Document(s)  <i>(if foreign priority is claimed)</i>          16. <input type="checkbox"/> Other: _____          _____          _____       </div>
---	---

17.	<b>If a CONTINUING APPLICATION</b> , check appropriate box and supply the requisite information: <input type="checkbox"/> Continuation <input type="checkbox"/> Divisional <input type="checkbox"/> Continuation-in-part (CIP) of prior application No.: Prior application information: Examiner: Group Art Unit:	
-----	---	--

<b>18. CORRESPONDENCE ADDRESS</b>					
NAME	David E. Brook, Esq.				
	HAMILTON, BROOK, SMITH & REYNOLDS, P.C.				
ADDRESS	Two Militia Drive				
CITY	Lexington	STATE	MA	ZIP CODE	02421-4799
COUNTRY	USA	TELEPHONE	(781) 861-6240	FAX	(781) 861-9540

Signature		Date	December 15, 1999
Submitted by Typed or Printed Name	Lisa M. Tréannie	Reg. Number	41,368

-1-

Date: <u>12-15-99</u>	Express Mail Label No. <u>EL192648508US</u>
-----------------------	---

Inventors: Leonard Guarente, Shin-ichiro Imai and Christopher Armstrong  
Attorney's Docket No.: 0050.1618000

METHODS FOR IDENTIFYING AGENTS WHICH ALTER  
HISTONE PROTEIN ACETYLATION,  
DECREASE AGING, INCREASE LIFESPAN

GOVERNMENT SUPPORT

- 5           The invention was supported, in whole or in part, by grant AG 11119 from the National Institutes of Aging. The Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

- Aging involves progressive and irreversible loss of cellular processes and physiological functions that ultimately increase the likelihood of death. Molecular  
10       correlates of aging, including an increase in chromosomal structural abnormalities, the frequency of single-strand DNA breaks, a decline in DNA methylation, and a loss of DNA telomeric sequences, have been described in a range of eukaryotic organisms from mammals, such as humans, to unicellular organisms, such as yeast.

- Although several mechanisms have been postulated as mediators of aging (e.g.,  
15       somatic mutation theory, error catastrophe theory, intrinsic DNA rearrangement theory), none have led to interventions or therapies to slow aging and increase life span. In humans, declining health in aging individuals has a significant impact on the cost and implementation of geriatric health care.

Thus, there is a need to identify agents which alter (e.g., agonize, antagonize) the level of substrates and cellular mediators associated with the aging process. The identification of such agents is important in the development of specific and effective treatment regimens to decrease aging or increase the life span of a cell or an organism, and to further define pathways which lead to aging in a cell or organism.

#### SUMMARY OF THE INVENTION

The present invention relates to methods of altering the NAD-dependent acetylation status of proteins, e.g., histone proteins, identifying agents which alter the NAD-dependent acetylation status of proteins, e.g., histone proteins, identifying agents which alter mono-ADP-ribosylation of nuclear proteins, alter aging or alter life span, as well as to methods of altering mono-ADP-ribosylation of nuclear proteins, methods of altering aging of a cell or organism, and methods of altering life span of a cell or organism. In preferred embodiments, the invention relates to methods of identifying agents which alter the NAD-dependent acetylation status of histone proteins by altering the activity of Sir2, increase mono-ADP-ribosylation of nuclear histone proteins, decrease aging or increase life span in a cell or organism, as well as to methods of increasing mono-ADP-ribosylation, decreasing aging or increasing life span of a cell or organism. In preferred embodiments, increasing mono-ADP-ribosylation or NAD-dependent deacetylation of histone proteins, decreasing aging or increasing life span of a cell or organism comprise administering to the cell or organism Sir2 or a mono-ADP-ribosyltransferase or an agonist of Sir2 and/or mono-ADP-ribosyltransferase activity, and combinations thereof.

In one embodiment, the method of the invention is a method of altering the NAD-dependent acetylation status of at least one amino acid residue in a histone protein by altering the activity of a Sir2 protein or a Sir2-like protein. The histone protein can be selected from the group consisting of a H2B, H3 or H4 histone protein. In a preferred embodiment, the amino acid residue is a lysine amino acid residue. In a particularly preferred embodiment, the lysine amino acid residue is lysine 9 and/or

lysine 14 of a H3 histone protein and/or lysine 16 of a H4 histone protein. The NAD-dependent acetylation status is removal of an acetyl group and/or addition of an acetyl group.

In a preferred embodiment, NAD-dependent acetylation status of the histone  
5 protein is altered by altering the activity of Sir2 $\alpha$  protein. In another embodiment, the NAD-dependent acetylation status is altered by altering the activity of a mutant Sir2 $\alpha$  protein selected from the group consisting of G253A, G255A, S257A, I262A, F265A, R266A, G270A, P285A, T336A, H355A, Thr-261, Iso-271, Arg-275, Asn-345 or Asp-347.

10 In another embodiment, the invention relates to a method of identifying an agent which alters the activity of a Sir2 protein or a Sir2-like protein by assessing the NAD-dependent acetylation status of at least one amino acid in a histone protein, comprising combining the histone protein, the Sir2 protein or the Sir2-like protein, NAD or a NAD-like compound and the agent to be tested, thereby producing a combination; detecting  
15 the NAD-dependent acetylation status of an amino acid in the histone protein; and comparing the NAD-dependent acetylation status in the presence of the agent to be tested with the NAD-dependent acetylation status of the amino acid in the histone protein in the absence of the agent to be tested, wherein a difference in the NAD-dependent acetylation status of the amino acid of the histone protein between the  
20 presence of the agent and the absence of the agent indicates that the agent alters the NAD-dependent acetylation status of at least one amino acid of the histone protein.

Another aspect of the invention relates to a method of identifying an agent which alters life span of a cell by assessing the NAD-dependent acetylation status of at least one amino acid in a histone protein, comprising combining the histone protein, a  
25 Sir2 protein or Sir2-like protein, NAD or a NAD-like compound and the agent to be tested, thereby producing a combination; detecting the NAD-dependent acetylation status of an amino acid in the histone protein; and comparing the NAD-dependent acetylation status in the presence of the agent to be tested with the acetylation status of the amino acid in the histone protein in the absence of the agent to be tested, wherein a



difference in the acetylation status of the amino acid of the histone protein between the presence of the agent alters the life span of the cell. The invention further relates to administering the agents identified by the method to a cell and assessing the NAD-dependent acetylation status of at least one amino acid in a histone protein of the cell.

- 5           In a further embodiment, the invention relates to a method of identifying an agent which alters the activity of a Sir2 protein or a Sir2-like protein by assessing the NAD-dependent acetylation status of at least one amino acid in a histone protein, comprising combining the histone protein, the Sir2 protein or the Sir2-like protein, NAD or a NAD-like compound and the agent to be tested, thereby producing a
- 10 combination; detecting the NAD-dependent acetylation status of an amino acid in the histone protein; and comparing the NAD-dependent acetylation status in the presence of the agent to be tested with the NAD-dependent acetylation status of the amino acid in the histone protein in the absence of the agent to be tested, wherein a difference in the NAD-dependent acetylation status of the amino acid of the histone protein between the
- 15 presence of the agent and the absence of the agent indicates that the agent alters the NAD-dependent acetylation status of at least one amino acid of the histone protein.

- In yet another embodiment, the invention relates to a method of identifying an agent which alters aging of a cell by assessing the NAD-dependent acetylation status of at least one amino acid in a histone protein, comprising combining the histone protein, a
- 20 Sir2 protein or Sir2-like protein, NAD or a NAD-like compound and the agent to be tested, thereby producing a combination; detecting the NAD-dependent acetylation status of an amino acid in the histone protein; and comparing the NAD-dependent acetylation status in the presence of the agent to be tested with the acetylation status of the amino acid in the histone protein in the absence of the agent to be tested, wherein a
- 25 difference in the acetylation status of the amino acid of the histone protein between the presence of the agent alters aging of the cell. In one embodiment, the agent increases aging of the cell. In another embodiment, the agent decreases aging of the cell.

          In another embodiment, the invention relates to a method of altering the NAD-dependent acetylation status of at least one amino acid residue in a histone protein

comprising combining the histone protein, a Sir2 protein or Sir2-like protein and a NAD or a NAD-like compound.

In yet another embodiment, the methods of the invention include methods for identifying an agent which alters mono-ADP-ribosylation of a nuclear protein in a cell  
5 or an organism, comprising combining a cell or organism and an agent to be tested; determining a level of mono-ADP-ribosylation of a nuclear protein in the cell or in one or more cells of the organism; and comparing the level of mono-ADP-ribosylation in the presence of the agent with the level of mono-ADP-ribosylation of the nuclear protein in the absence of the agent. A difference in the level of mono-ADP-ribosylation  
10 of the nuclear protein in the presence of the agent as compared with in the absence of the agent indicates that the agent alters mono-ADP-ribosylation of the nuclear protein. In a preferred embodiment, the agent is an agonist of mono-ADP-ribosylation. In another embodiment, the agent is an antagonist of mono-ADP-ribosylation.

In another embodiment, the invention relates to methods for identifying an agent  
15 which alters life span of a cell or an organism, comprising combining a cell or organism and an agent to be tested; determining a level of mono-ADP-ribosylation of a nuclear protein in the cell or in one or more cells of the organism; and comparing the level of mono-ADP-ribosylation in the presence of the agent with the level of mono-ADP-ribosylation of the nuclear protein in the absence of the agent. A difference in the level  
20 of mono-ADP-ribosylation of the nuclear protein in the presence of the agent as compared with in the absence of the agent indicates that the agent alters mono-ADP-ribosylation of the nuclear protein, wherein agents which alter mono-ADP-ribosylation of nuclear proteins are agents which alter life span of a cell or an organism. In a preferred embodiment, the agent is an agonist of mono-ADP-ribosylation. In another  
25 embodiment, the agent is an antagonist of mono-ADP-ribosylation.

The invention also relates to a method of identifying an agent which increases life span of a cell or an organism, comprising the steps of combining a cell or organism and an agent to be tested; determining a level of mono-ADP-ribosylation of a nuclear protein in the cell or in one or more cells of the organism in the presence of the agent

and in the absence of the agent; identifying an agent which increases mono-ADP-ribosylation of a nuclear protein; administering said agent to a cell or an organism; and determining the life span of said cell, wherein an agent which increases the life span of said cell or organism relative to the mean life span of said cell or organism or relative to  
5 the life span of said cell or organism in the absence of the agent is an agent which increases life span of a cell or organism.

The invention also relates to a method of identifying an agent which decreases life span of a cell or an organism, comprising the steps of combining a cell or organism and an agent to be tested; determining a level of mono-ADP-ribosylation of a nuclear  
10 protein in the cell or in one or more cells of the organism in the presence of the agent and in the absence of the agent; identifying an agent which decreases mono-ADP-ribosylation of a nuclear protein; administering said agent to a cell or an organism; and determining the life span of said cell, wherein an agent which decreases the life span of said cell or organism relative to the mean life span of said cell or organism or relative to  
15 the life span of said cell or organism in the absence of the agent is an agent which decreases life span of a cell or organism.

Also encompassed by the present invention is a method of identifying an agent which alters aging of a cell, comprising the steps of combining a cell and an agent to be tested; determining a level of mono-ADP-ribosylation of a nuclear protein in the cell;  
20 and comparing the level of mono-ADP-ribosylation in the presence of the agent with a level of mono-ADP-ribosylation of the nuclear protein in the absence of the agent to be tested. A difference in the level of mono-ADP-ribosylation of the nuclear protein between the presence of the agent and the absence of the agent indicates that the agent alters aging of the cell. In particular embodiment, the agent decreases aging of the cell.  
25 In another embodiment, the agent increases aging of the cell.

Another aspect of the invention includes a method of increasing the life span of a cell, comprising administering to the cell an effective amount of an agent which increases mono-ADP-ribosylation of a nuclear protein. The agent to be administered is identified by a method comprising the steps of combining a cell and an agent to be

tested; determining a level of mono-ADP-ribosylation of a nuclear protein in the cell; and comparing the level of mono-ADP-ribosylation in the presence of the agent with a level of mono-ADP-ribosylation of the nuclear protein in the absence of the agent to be tested, wherein in the presence of the agent there is an increase in the level of mono-ADP-ribosylation of the nuclear protein.

The invention also relates to a method of decreasing aging of a cell, comprising administering to the cell an effective amount of an agent which increases mono-ADP-ribosylation of a nuclear protein. The agent to be administered is identified by a method comprising the steps of combining a cell and an agent to be tested;

10 determining a level of mono-ADP-ribosylation of a nuclear protein in the cell; and comparing the level of mono-ADP-ribosylation in the presence of the agent with a level of mono-ADP-ribosylation of the nuclear protein in the absence of the agent to be tested, wherein in the presence of the agent there is an increase in the level of mono-ADP-ribosylation of the nuclear protein.

15 The invention further pertains to a method of increasing the life span of a cell or an organism comprising administering to the cell or organism a mono-ADP-ribosyltransferase or an agonist of mono-ADP-ribosyltransferase activity in an amount effective to increase the life span of the cell or organism.

In yet another embodiment, the present invention pertains to a method of  
20 increasing the life span of a cell or organism comprising administering to the cell or organism an agonist of mono-ADP-ribosylation of histone protein H2B in an amount effective to increase the life span of the cell or organism.

Another embodiment of the invention pertains to a method of decreasing aging of a cell or organism comprising administering to the cell or organism a mono-ADP-  
25 ribosyltransferase or an agonist of mono-ADP-ribosyltransferase activity in an amount effective to decrease aging of the cell or organism.

Also encompassed by the present invention is a method of decreasing aging of a cell or organism comprising administering to the cell or organism an agonist of mono-

ADP-ribosylation of histone protein H2B in an amount effective to decrease aging of the cell or organism.

The invention also relates to a method of inhibiting the formation, replication and/or accumulation of rDNA circles in a cell comprising administering to the cell a  
5 mono-ADP-ribosyltransferase or an agonist of mono-ADP-ribosyltransferase activity in an amount effective to inhibit the formation, replication and/or accumulation of rDNA circles.

Another aspect of the invention is a method for decreasing recombination between rDNA in a cell comprising administering to the cell a mono-ADP-  
10 ribosyltransferase or an agonist of mono-ADP-ribosyltransferase activity in an amount effective to decrease recombination between rDNA.

In a preferred embodiment, the nuclear protein in the methods of the present invention is selected from the group consisting of histone proteins H2A, H2B and H3. In particular, the mono-ADP-ribosylation of nuclear proteins is performed by the core  
15 domain of Sir2, the Sir2 protein, a fragment of the Sir2 protein (e.g., SEQ ID NOS: 1, 2, 4, 9, 10, 11, 12, 14, 19, 20, 21 or 26), or an agonist of Sir2.

In one embodiment of the methods described herein, the cell is a yeast cell. In another embodiment, the cell is a mammalian cell.

The present invention also provides an isolated murine Sir2 protein, preferably  
20 the murine Sir2 $\alpha$  protein (SEQ ID NO: 26) and the nucleic acid sequence encoding the Sir2 $\alpha$  (SEQ ID NO: 25).

The invention also pertains to isolated nucleic acid molecules of murine SIR2 genes operably linked to a regulatory sequence and methods of preparing Sir2 proteins by culturing the recombinant host cells comprising isolated nucleic acid molecules of  
25 murine SIR2 genes.

Also encompassed by the present invention, are antibodies, or antigen-binding fragments thereof, which selectively bind a murine Sir2 proteins or a murine Sir2-like protein.

The invention described herein provides novel methods to readily identify agents that alter the NAD-dependent acetylation status of nuclear proteins and/or the mono-ADP-ribosylation of a nuclear protein; and to utilize the identified agents to alter NAD-dependent acetylation, ADP-ribosylation, aging and increase life span. For example,  
 5 the invention provides unique and efficient ways to forestall senescence and extend life in cells and organisms.

### BRIEF DESCRIPTION OF THE FIGURES

Figure 1A is a diagrammatic representation of the phylogenetic tree of yeast and murine Sir2 protein core domains.

10 Figure 1B is a Northern blot analysis of the tissue distribution of murine SIR2 $\alpha$ ,  $\beta$ , and  $\gamma$  mRNA transcripts.

Figure 2A is the deduced amino acid sequence of mSir2 $\alpha$  (SEQ ID NO: 1) with the predicted nuclear localization signal underlined.

Figure 2B is a Western blot of the mSir2 $\alpha$  protein (120kD) immunoprecipitated  
 15 (IP) from cell extracts of murine NIH3T3 cells and *in vitro* translated (IVF) probed with a polyclonal antibody against the N-terminal 131 amino acids of the protein ( $\alpha$ mSir2 $\alpha$ ).

Figure 2C is an alignment of the evolutionarily conserved core domains of yeast (ySir2, GenBank Accession Nos: X01419, M21316, SEQ ID NO: 2; yHST1, GenBank Accession Nos: U39041, L47120, SEQ ID NO: 3); murine (mSir2 $\alpha$ , SEQ ID NO: 4 )  
 20 and *Salmonella* (CobB, GenBank Accession No: U89687, SEQ ID NO: 5) Sir2 proteins. Identical amino acids are boxed. A putative NAD binding cleft is indicated by asterisks. Less conserved amino acids are shaded.

Figure 2D is a schematic alignment of the core domain of mSir2 $\alpha$ , ySir2p and CobB proteins.

25 Figure 3 depicts the immunocytochemical localization of mSir2 $\alpha$  in mouse NIH3T3 cells (A, D, G, and J). Cells were counterstained with DAPI (B, E, H, and K). Nucleoli (E), telomeres (H), and centromeres (K) were visualized using an anti-human

nucleolar antibody (ANA-N), telomeric FISH, and an anti-human centromeric antibody (ANA-C), respectively. Merged images are shown in C, F, I, and L.

Figure 4A is a Coomassie blue stained gel of 6XHis-tagged recombinant yeast (r-y Sir2p) and murine (r-m Sir2 $\alpha$ ) Sir2 proteins purified with Ni-NTA agarose under native conditions. Arrowheads indicate each full-length protein.

Figure 4B depicts the results of ADP-ribosylation of histone proteins H1, H2A, H2B, H3 and H4 by recombinant r-ySir2p and r-mSir2 $\alpha$ .

Figure 4C illustrates ADP-ribosyltransferases cleaving nicotinamide (Nam) from NAD<sup>+</sup> (Ade-Rib-P\*) creating a linkage between ribose (Rib) and histone proteins.

Figure 4D illustrates the dose-dependent modification of H2B and H3 by recombinant yeast r-ySir2p and murine r-mSir2 $\alpha$  proteins.

Figure 4E illustrates the effects of snake venom phosphodiesterase (SVP) on the removal of radiolabeled <sup>32</sup>P-NAD from Sir2-modified H2B and H3.

Figure 4F depicts the effects of mono-ADP-ribosylation inhibitors (novobiocin and coumermycin A1), but not poly-ADP-ribosylation inhibitors (3-aminobenzamide (3-ABA) and benzamide), on mSir2 $\alpha$  ADP-ribosylation of H2B.

Figure 4G depicts the mono-ADP-ribosyltransferase activity of mSir2 $\alpha$  immunoprecipitated by an antiserum to mSir2 $\alpha$  ( $\alpha$ mSir2 $\alpha$ ) from NIH3T3 whole cell extracts.

Figure 5A depicts the amino acid sequences of the N-terminal tails of H3 (SEQ ID NO: 6) and H4 (mono Ac, SEQ ID NO: 7, tetra Ac, SEQ ID NO: 8) peptides. The asterisks depict the site of acetylation (mono Ac, tetra Ac) to generate peptides with and without acetylated lysines. Human H3 GenBank Accession No: M26150, mouse H3 GenBank Accession Nos: M23459, 32460, 32461, 32462. Human H4 GenBank Accession Nos: M60749, M16707, mouse H4 GenBank Accession No: U62672.

Figure 5B depicts the effects of mSir2 $\alpha$  and ySir2p on diacetylated H3 peptide (diAc), but not unacetylated H3 (unAc). A bracket, an arrowhead, and an arrow at right indicate modified peptides, hydrolyzed and/or decomposed products of <sup>32</sup>P-NAD, and unhydrolyzed <sup>32</sup>P-NAD, respectively.

Figure 5C illustrates mSir2 $\alpha$  modification of Lys16-acetylated H4 peptide (Ac16). Unacetylated (unAc), monoacetylated (Ac5, Ac8, Ac12, or Ac16), and tetraacetylated (tetraAc) peptides were added in the reactions with the control eluate (pET) or the mSir2 $\alpha$ , respectively.

- 5        Figure 6A depicts the highly conserved amino acid residues in the core domain of mSir2 $\alpha$  (SEQ ID NO: 9) and ySir2 (SEQ ID NO: 10) proteins. Identical amino acids are boxed. Arrowheads depict the amino acid residues used to generate mutant Sir2 proteins which were evaluated for ADP-ribosylation activity.

- Figure 6B depicts the ADP-ribosylation activities of mSir2 $\alpha$  proteins (Wildtype, WT; vector alone, pET; mutants G253A, G255A, S257A, I262A, F265A, R266A, G270A, P285A, T336A, H355A) on histones H2B and H3.
- 10        WT; vector alone, pET; mutants G253A, G255A, S257A, I262A, F265A, R266A, G270A, P285A, T336A, H355A) on histones H2B and H3.

Figure 6C illustrates the quantitation of the ADP-ribosylation activities of the mSir2 $\alpha$  mutants (G253A, G255A, S257A, I262A, F265A, R266A, G270A, P285A, T336A, H355A) compared to wild type (WT) and control pET.

- 15        Figure 6D illustrates the *in vivo* transcriptional repression by the mSir2 $\alpha$  wildtype and mutant core domain fused to the GAL4 DNA binding domain (DBD).

Figure 7 is a diagrammatic representation of a model of Sir2-mediated modification of histone proteins H3 and H4.

- Figures 8a, 8b, 8c, 8d, 8e, 8f, and 8g illustrate the *in vitro* deacetylation of the H3 peptide (residues 1-20) di-acetylated at lysines 9 and 14 by recombinant yeast Sir2p.
- 20        H3 peptide (residues 1-20) di-acetylated at lysines 9 and 14 by recombinant yeast Sir2p.

Figure 8a is a Coomassie blue-stained SDS-PAGE gel of purified recombinant yeast (rSir2p) protein, mouse Sir2 (mSir2 $\alpha$ ) proteins and vector controls. Full length proteins are indicated by dots.

- Figures 8b, 8c, 8d, 8e and 8f are HPLC chromatograms showing absorbance at 220 nm of products of deacetylation assays with yeast Sir2p and the indicated concentrations of NAD. The efficiencies of the reactions are calculated from the areas under peaks 1, 2, and 4.
- 25        220 nm of products of deacetylation assays with yeast Sir2p and the indicated concentrations of NAD. The efficiencies of the reactions are calculated from the areas under peaks 1, 2, and 4.

Figure 8g is a schematic of contents of peaks 1-5 shown in chromatograms.



Figures 9a, 9b, 9c and 9d are electron-spray mass spectroscopy of peaks 3-5 of HPLC chromatogram.

Figures 9a and 9b depict peak 3 of the HPLC chromatogram of the deacetylase reaction at 1mM NAD. The molecular weights of the two products in peak 3 correspond to the starting peptide (2370) and the doubly deacetylated dimer (2-times the molecular weight of the starting peptide, 4740, minus the difference between the molecular weight of two acetyl moieties versus two hydrogens, 84, yielding 4656).

Figure 9c Peak 4 of the same reaction. The molecular weight of peak 4 (4698) corresponds to singly deacetylated dimer peptide.

Figure 9d Peak 5 of the same reaction corresponds to dimer peptide (molecular weight 4740).

Figures 10a, 10b, 10c, 10d, 10e and 10f are the amino-terminal sequencing of peaks 4 and 5 of the deacetylase reaction at 1 mM NAD. Peaks 4 and 5 were subjected to sequencing by Edmann degradation. Chromatograms at positions 9, 14, and 18 are shown. In peak 4, about 23% of Lys9 (Figure 10a) and 27% of Lys 14 (Figure 10b) were deacetylated. In peak 5, both Lys 9 and 14 are essentially all acetylated (Figure 10d and Figure 10e). The unacetylated Lys18 of both peaks 4 and 5 are shown for comparison (Figure 10c and Figure 10f). The peak to the right of the acetylated lysine at position 14 corresponds to alanine, which is a preview of Ala 15.

Figures 11a, 11b, 11c, 11d and 11e illustrate the effects of inhibitors on the deacetylase and putative ADP-ribosyltransferase activities of recombinant Sir2p (rSir2p). The HPLC chromatograms of the reactions in the presence of solvent only (Figure 11a), 400nM TSA (Figure 11b) and 200μM coumermycin A1 (Coumer) (Figure 11e) are shown. The calculated efficiencies of the reactions are indicated. The effect of 200μM coumermycin A1 on ADP-ribosylation of the intact histone H3 (Figure 11c) and the H3 peptide (Figure 11d) are examined on SDS-PAGE and TLC, respectively. pET corresponds to the vector control.

Figure 11d shows the ADP-ribosylated peptide indicated by a bracket on a longer exposure (left), and NAD and its hydrolysed product are indicated by an arrow and an arrowhead on a shorter exposure, respectively (right).

Figures 12a, 12b, and 12c show the deacetylation activity of the mouse Sir2p homolog, mSir2 $\alpha$ .

Figure 12a is the amino acid sequence of mSir2 $\alpha$  (SEQ ID NO: 1). The evolutionarily conserved core domain of mSir2 $\alpha$  is boxed.

Figure 12b show the phylogenetic tree of yeast and mouse Sir2 core domains. The core domain sequences of three mouse homologs termed  $\alpha$ ,  $\beta$ , and  $\gamma$  are compared on Clustal X and NJPLOT programs to generate the phylogenetic tree.

Figure 12c illustrates the HPLC chromatogram of the product of deacetylation assay with 10 $\mu$ g of recombinant mSir2 $\alpha$  protein at 1mM NAD. The calculated efficiency of the reaction is indicated.

Figures 13a, 13b, 13c, 13d and 13e illustrate the effects of NAD derivatives on the Sir2p deacetylation activity of the H3 peptide. The HPLC chromatograms of the reactions with no NAD derivatives (Figure 13a) and 1mM concentration of NAD (Figure 13b), NADH (Figure 13c), NADP (Figure 13d), and NADPH (Figure 13e) are shown. The calculated efficiencies of the reactions are also indicated.

Figures 14a, 14b and 14c depict the putative ADP-ribosylation activities of Sir2 protein core domain mutants.

Figure 14a is the amino acid sequence of the core domains of ySir2p (SEQ ID NO: 11), mSir2 $\alpha$  (SEQ ID NO: 12) and CobB (SEQ ID NO: 13) aligned and six highly conserved residues, indicated by arrowheads, were mutated to alanine.

Figure 14b shows the 6XHis tagged versions of wildtype ySir2p (wt) and the six mutant Sir2p (Thr-261, Gly-270, Iso-271, Arg-275, Asn-345, Asp-347) and a vector control (vector) expressed in *E. coli*, purified over a Nickel-NTA column and analyzed on a 7% polyacrylamide SDS gel to assess expression levels.

Figure 14c depicts the ability of ySir2p and mutant Sir2p proteins to modify histone H3 with  $^{32}$ P labeled NAD.

Figures 15a, 15b, 15c, 15d, 15e, 15f, 15g and 15h illustrate the effects of mutations in ySir2p on NAD dependent deacetylation of H3. The peptides were run on HPLC and the chromatograms based on the absorption at 220 nm recorded. The efficiency of reactions were determined by summing the area under the appropriate peaks.

Figures 16a, 16b, 16c and 16d illustrate the effect of mutations on deacetylation *in vitro* and silencing *in vivo*. Wildtype (wt) and mutant Sir2 (Thr-261, Gly-270, Iso-271, Arg-275, Asn-345) were integrated into *sir2Δ* W303R strains.

Figure 16a is a Western blot of 25 μg of whole cell extract from wildtype, *sir2Δ* and Sir2 mutants probed with Sir2 antibody. The upper band corresponds to Sir2p and a lower background band is included as a loading control.

Figure 16b illustrates silencing at HMLα by mating the strains with tester strains of the opposite mating type and monitoring growth of diploids on selective media.

Figure 16c illustrates telomere-silencing by the ability of strains with telomeric *URA3* to grow on media containing 5-FOA, which is toxic when *URA3* is expressed, but harmless when *URA3* is silenced.

Figure 16d illustrates rDNA silencing was monitored in a strain with the *ADE2* marker located within the rDNA array. *RPD3* was disrupted to enhance silencing differences between the wildtype and *sir2Δ* strains. *sir2* mutant strains were spotted on complete and -ade media to monitor effects on silencing.

Figures 17a, 17b and 17c illustrate the effect of mutations in Sir2 on rDNA recombination and yeast life span.

Figure 17a illustrates W303R with *ADE2* at rDNA tested for rDNA recombination rates by counting the frequency of loss of the marker in the first generation after plating.

Figure 17b illustrates the life span of wildtype, *sir2Δ*, and mutants 261, 270, and 275 measured in *hmlΔ* strains where the a/α effects are eliminated.

Figure 17c illustrates the life span of wildtype, *sir2Δ*, and mutants 261 and 270 measured in *HML+* strains as a sensitive test of *a/α* silencing.

Figure 18 is a summary of *in vitro* and *in vivo* data regarding ADP-ribosyltransferase, deacetylase, HM silencing, telomere silencing, rDNA silencing, rDNA recombination and mean life span for Sir2 wildtype and mutant Sir2 proteins (Thr-261, Gly-270, Iso-271, Arg-275, Asn-345, Asp-347).

Figure 19 depicts the amino acid sequence alignment of the core domain of ySir2 (SEQ ID NO: 14), yHST1 (SEQ ID NO: 15), yHST2 GenBank Accession NO: U39063, (SEQ ID NO: 16), yHST3 GenBank Accession No: U39062, (SEQ ID NO: 17), yHST4 GenBank Accession No: NC\_001136 (SEQ ID NO: 18), mSir2alpha (mSir2 $\alpha$ , SEQ ID NO: 19), mSir2beta (mSir2 $\beta$ , SEQ ID NO: 20), mSirg (mSir2 $\gamma$ , SEQ ID NO: 21), and deduced amino acid sequences of Sir2-like core domains (GenBank Accession No: A1465098, SEQ ID NO: 22; GenBank Accession No: A1465820, SEQ ID NO: 23; GenBank Accession No: A1466061, SEQ ID NO: 24).

Figure 20 depicts the effects of multiple copies of Sir2 on yeast life span.

Figures 21a, 21b, 21c, and 21d depict the nucleotide sequence (GenBank Accession No: AF214646, SEQ ID NO: 25) and amino acid sequence (SEQ ID NO: 26) of murine Sir2 $\alpha$ .

Figure 22 depicts the nucleotide sequence (SEQ ID NO: 34) and amino acid sequence (SEQ ID NO: 35) of an EST CDNA clone 557657 (Genome Systems, Inc.).

## DETAILED DESCRIPTION OF THE INVENTION

The invention relates to the discovery that Sir2 proteins, which play a part in the life span control mechanisms in eukaryotic cells, alter the NAD-dependent acetylation status of histone proteins and mono-ADP-ribosylate nuclear proteins. In particular, the invention pertains to the discovery that Sir2 (e.g., murine Sir2 $\alpha$ ) alters the NAD-dependent acetylation status (e.g., removes and/or adds an acetyl group) of histone proteins (e.g., histone proteins H2B, H2A, H3 and/or H4) and mono-ADP-ribosylates histone proteins. As a result of this discovery, methods for identifying agents (e.g.,

agonists) which alter the NAD-dependent acetylation status of nuclear proteins, such as histone proteins, and/or their mono-ADP-ribosylation are available to provide methods and compositions to alter (e.g., slow) aging and alter (e.g., increase) life span of cells and organisms.

- 5           The present invention relates to a method of altering the NAD-dependent acetylation status of at least one amino acid residue of a histone protein by altering the activity of a Sir2-protein or Sir-2 like protein. In a particular embodiment, the NAD-dependent acetylation status of H3 and/or H4 is altered. Specifically encompassed by the invention is the alteration of lysine residues in the N-terminus of H3 (e.g., lysine 9  
10 and/or 14) and H4 (e.g., lysine 16).

- “NAD-dependent” as used herein refers to a requirement for NAD (nicotinamide adenine dinucleotide) compound in a reaction. The reaction can performed or take place in the presence (e.g., *in vivo*) or the absence (e.g., *in vitro*) of cells. A “NAD-like compound” is also within the scope of the invention and refers to a compound (e.g., a  
15 synthetic or naturally occurring chemical, drug, protein, peptide, small organic molecule) which possesses structural similarity (e.g., adenine, ribose and phosphate groups) or functional similarity (e.g., oxidation of substrates, NAD-dependent deacetylation of histone proteins). For example, NAD-like compounds can be NADH, NADP, NADPH, non-hydrolyzable NAD and fluorescent analogs of NAD (e.g., 1, N6-  
20 etheno NAD).

- The term “NAD-dependent acetylation status” refers to the requirement of NAD to either transfer (also referred to herein as the addition) or remove (also referred to herein as deacetylation) at least one acetyl group (e.g.,  $\text{CH}_3\text{CO--}$ ) to a substrate having OH or  $\text{NH}_2$  groups (e.g., at least one amino acid residue of a histone protein such as  
25 H2B, H2A, H3 and/or H4). Thus, “acetylation status” can be either acetylation or deacetylation of a substrate.

          In a particular embodiment, the amino acid residue who acetylation status is altered is a basic amino acid (e.g., lysine, arginine, histidine). In a preferred embodiment, the acetylation status lysine residues of histone proteins H1, H2A, H2B,

H3 or H4 are altered. In a more preferred embodiment, the acetylation status of lysine residues at the amino terminus of H3 and H4 is altered. In particular, lysine 9 and/or lysine 14 of H3 and/or lysine 16 of H4. Any suitable amino acid residue (e.g. having OH or NH<sub>2</sub> groups) capable of undergoing an alteration in acetylation status in a cell and/or an animal or under *in vitro* conditions (e.g., outside a cell or an animal) is within the scope of the invention.

The invention also relates to a method of identifying an agent which alters the activity of a Sir2 protein or a Sir2-like protein by assessing the NAD-dependent acetylation status of at least one amino acid in a histone protein, comprising combining the histone protein, the Sir2 protein or the Sir2-like protein, NAD or a NAD-like compound and the agent to be tested, thereby producing a combination; detecting the NAD-dependent acetylation status of an amino acid in the histone protein; and comparing the NAD-dependent acetylation status in the presence of the agent to be tested with the NAD-dependent acetylation status of the amino acid in the histone protein in the absence of the agent to be tested, wherein a difference in the NAD-dependent acetylation status of the amino acid of the histone protein between the presence of the agent and the absence of the agent indicates that the agent alters the NAD-dependent acetylation status of at least one amino acid of the histone protein.

The agent identified by the methods of the invention can add an acetyl group or remove an acetyl group from at least one amino acid residue (e.g., lysine) of a histone protein (e.g., H2B, H3 or H4).

The invention further relates to a method of identifying an agent which alters life span of a cell by altering the activity of a Sir2 protein or a Sir2-like protein by assessing the NAD-dependent acetylation status of at least one amino acid (e.g., lysine) in a histone protein (e.g., H2B, H3 or H4). The histone protein, a Sir2 protein or Sir2-like protein, NAD or a NAD-like compound and the agent to be tested are combined to produce a combination, the NAD-dependent acetylation status of an amino acid in the histone protein is detected (e.g., electron-spray or matrix assisted laser desorption/ionization (MALDI) mass spectroscopy); and compared the NAD-dependent

acetylation status in the presence of the agent to be tested with the acetylation status of the amino acid in the histone protein in the absence of the agent to be tested. A difference in the acetylation status of the amino acid of the histone protein between the presence and absence of the agent alters the life span of the cell. The agent tested can  
5 increase the lifespan of a cell by NAD-dependent deacetylation of the histone protein. Alternatively, or additionally, the agent can decrease the lifespan of the cell by NAD-dependent acetylation of histone proteins. The agent can be an agonist of Sir2 activity or the agent can be an antagonist of Sir2 activity.

The invention also provides a method of identifying an agent which alters aging  
10 of a cell by altering the activity of Sir2 or a Sir-2 like protein by assessing the NAD-dependent acetylation status of at least one amino acid (e.g., lysine) in a histone protein (e.g., H2B, H3 or H4). The histone protein, a Sir2 protein or Sir2-like protein, NAD or a NAD-like compound and the agent to be tested are combined to produce a combination, the NAD-dependent acetylation status of an amino acid in the histone  
15 protein is detected (e.g., electron-spray mass spectroscopy); and compared the NAD-dependent acetylation status in the presence of the agent to be tested with the acetylation status of the amino acid in the histone protein in the absence of the agent to be tested. A difference in the acetylation status of the amino acid of the histone protein in the presence of the agent alters aging of the cell. The agent tested can increase the aging of  
20 a cell by NAD-dependent acetylation of the histone protein. Alternatively, the agent can decrease aging of the cell by NAD-dependent deacetylation of histone proteins. The agent can be an agonist of Sir2 or an antagonist of Sir2.

Agents identified by the *in vitro* methods of the invention, such as the method described above, can be further evaluated for their ability to alter the NAD-dependent  
25 acetylation status in a cell by administering the identified agents to a cell and monitoring the NAD-dependent acetylation status of at least one amino acid in a histone protein of the cell. It is expected that agents identified *in vitro* as agents which alter the NAD-dependent acetylation status of a histone protein will alter the NAD-dependent alteration status of histone protein *in vivo*. Likewise, it is expected that the agents which alter

NAD-dependent acetylation status of histone proteins by the methods described herein will alter the lifespan and aging of a cell and/or organism.

The invention also relates to a method of altering the NAD-dependent acetylation status of at least one amino acid residue in a histone protein comprising  
5 combining the histone protein, a Sir2 protein or Sir2-like protein and a NAD or a NAD-like compound. The method can be used *in vitro* (e.g., the absence of cells or an animal) or *in vivo* (e.g., in cells or animals).

The present invention also relates to a method of identifying agents which alter mono-ADP-ribosylation of one or more nuclear proteins in a cell. The cell and agent to  
10 be tested are combined and the level of mono-ADP-ribosylation of a nuclear protein, such as one or more histone proteins (e.g., H2B, H2A, H3, H4), is determined and compared to the level of mono-ADP-ribosylation of that protein or proteins in the absence of the agent. A difference in the level of mono-ADP-ribosylation in the presence and absence of the agent indicates that the agent alters the mono-ADP-  
15 ribosylation of the nuclear protein. That is, if the level of mono-ADP-ribosylation is greater in the presence of the agent than in the absence of the agent, the agent is an agonist of mono-ADP-ribosylase activity. Similarly, if the level of mono-ADP-ribosylation is greater in the absence of the agent than in the presence of the agent, the agent is an antagonist of mono-ADP-ribosylation.

20 Alternatively, the method of identifying agents which alter NAD-dependent acetylation status of a histone protein and/or mono-ADP-ribosylation of one or more nuclear proteins can be carried out at the organism (e.g. animal) level. An agent to be tested is administered to an organism, and the alteration in NAD-dependent acetylation of a histone protein and/or the level of mono-ADP-ribosylation of one or more nuclear  
25 proteins, such as one or more histone proteins, in cells of the organism is determined and compared to the acetylation status of a histone protein and/or the level of mono-ADP-ribosylation of that protein or proteins in the absence of the agent. A difference in the acetylation status of a histone protein and/or the level of mono-ADP-ribosylation in the presence and absence of the agent indicates that the agent alters NAD-dependent



acetylation status of histone proteins and/or the mono-ADP-ribosylation of the nuclear protein.

Agents to be tested for activity in the assays described herein can include proteins (including post-translationally modified proteins), peptides (including  
5 chemically or enzymatically modified peptides), or small molecules (including carbohydrates, steroids, lipids, anions or cations, drugs, small organic molecules, oligonucleotides, antibodies, and genes encoding proteins of the agents or antisense molecules), including libraries of compounds. The agents can be naturally occurring (e.g., found in nature or isolated from nature) or can be non-naturally occurring (e.g.,  
10 synthetic, chemically synthesized or man-made). Agents which alter the level of NAD-dependent acetylation status of histone proteins or the mono-ADP-ribosylation of nuclear proteins of the invention can be agonists (e.g., stimulators/enhancers) or antagonists (e.g., inhibitors) of NAD-dependent acetylation or mono-ADP-ribosylase activity. In a particular embodiment, the agents are agonists or antagonists of Sir2 (e.g.,  
15 mSir2 $\alpha$ , ySir2) dependent NAD-dependent acetylation and mono-ADP-ribosylation of histone proteins (e.g., H2A, H2B, H3, H4).

The term "agonist" as used herein, refers to an agent which simulates or mimics NAD-dependent acetylation or deacetylation of histone proteins (e.g., mSir2, ySir2, human Sir2), a mono-ADP-ribosylase (e.g., mSir2 $\alpha$ , ySir2, human Sir2) by, for  
20 example, deacetylating H3 or H4 (e.g., removal an acetyl group from at least one lysine residue in the amino terminus) and/or catalyzing the transfer of an adenosine diphosphate ribose unit from an ADP donor (e.g., NAD) to an amino acid residue (e.g., lysine) of a substrate (e.g., histone protein H2B, H3, H4). Alternatively, or additionally, an agonist can be an agent which stimulates, augments, enhances, increases, intensifies  
25 or strengthens NAD-dependent acetylation/deacetylation and/or mono-ADP-ribosylase activity or the interaction between a nuclear protein and a NAD-dependent acetylase, mono-ADP-ribosyltransferase, or alters a mono-ADP-ribosylase in a manner (e.g., results in a conformational change) which augments the activity of the mono-ADP-ribosylase such that the nuclear protein is mono-ADP-ribosylated at a higher rate or in

greater quantities than in the absence of the agonist. An agonist can also enhance the rate of removal of acetyl groups from amino acid residues of histone proteins.

An agonist can also enhance the availability or accessibility of a mono-ADP-ribosylase to a substrate, or a substrate to a mono-ADP-ribosylase, thereby further  
5 increasing the level of mono-ADP-ribosylation of a nuclear protein. For example, a substance possessing agonist activity can increase the rate at which ADP-ribose is transferred to an amino acid residue of a histone protein, such as H2B, H2A, H3, or H4 beyond that observed in the absence of the agonist.

The term “antagonist”, as used herein, includes a substance which blocks,  
10 diminishes, inhibits, hinders, limits, decreases, reduces, restricts or interferes with the NAD-dependent acetylation/deacetylation of histone proteins and/or enzymatic activity of a mono-ADP-ribosylase, alters the substrate (e.g., a histone protein such as H2B, H2A, H3 or H4). An antagonist can act in a manner which prevents Sir2 from deacetylating an amino acid residue in a histone protein and/or a mono-ADP-ribosylase  
15 (e.g., mSir2 $\alpha$ , ySir2, human Sir2) from acting on the substrate, or interferes with the accessibility of the substrate (e.g., H2B, H2 or H4) to Sir2 for alterations in NAD-dependent acetylation status and/or the mono-ADP-ribosyltransferase, or any combination thereof.

Alternatively or additionally, an antagonist can prevent, impede or interfere with  
20 the interaction between Sir2 and NAD, a mono-ADP-ribosyltransferase and a nuclear protein substrate by altering the Sir2 or mono-ADP ribosyltransferase (e.g., conformationally), thereby preventing the Sir2 and/or a mono-ADP-ribosyltransferase from altering the NAD-dependent acetylation status of a histone protein and/or catalyzing the transfer of an adenosine diphosphate ribose unit from an ADP donor (e.g.,  
25 NAD) to an amino acid residue (e.g., threonine) of a substrate (e.g., a nuclear protein such as a histone protein). For example, a substance possessing antagonist activity can decrease the rate at which the status of NAD-dependent acetylation (e.g., deacetylation) takes place.

An agonist can also prevent, impede or interfere with ADP-ribose transfer to an amino acid residue of a histone protein, such as H2B, H2A, H3 or H4, relative to that observed in the absence of the antagonist.

The agonist or antagonists of the present invention can also alter transcription of NAD-dependent acetylases and/or mono-ADP-ribosyltransferase genes (e.g., SIR2), the mRNA stability of NAD-dependent acetylases and/or mono-ADP-ribosyltransferase transcripts, or degradation of the enzyme as a means to alter NAD-dependent acetylase and/or mono-ADP-ribosyltransferase activity. For example, an agonist can increase the level (e.g., total cellular pool) of deacetylated histone proteins by increasing the activity of Sir2 in an NAD-dependent manner. Likewise agonists of the invention can also mono-ADP-ribosylated nuclear protein by increasing transcription of mono-ADP-ribosyltransferase enzyme genes (e.g., SIR2 $\alpha$ ) or decreasing enzyme degradation in a cell.

Similarly, an antagonist can decrease the levels of deacetylated histone proteins or increase the amount of acetylated histone proteins. Antagonists can also decrease the levels of mono-ADP-ribosylation of a nuclear protein by down regulating transcription of mono-ADP-ribosyltransferase genes or increasing degradation of the enzyme.

As used herein, a "cell" refers to a single cell or a collection of at least two or more cells. The cell can be a yeast cell (e.g., *Saccharomyces cerevisiae*) or a mammalian cell, including but not limited to, somatic or embryonic cells, Chinese hamster ovary cells, HeLa cells, human 293 cells and monkey COS-7 cells. The collection of cells can form a tissue or an organism. An "organism" refers to any living individual, such as a vertebrate animal (e.g., a mammal, including but not limited to, mice, rats, pigs dogs, cats, primates, and humans ) or nonvertebrate animal, such as an insect (e.g., *Drosopholia melanogaster*) or nematode (e.g., *C. elegans*), considered as a whole. A "tissue" refers to a collection of similar cell types (such as epithelium, connective, muscle and nerve tissue).

The term "mono-ADP-ribosylation", as used herein, refers to the transfer of one adenosine diphosphate ribose unit from an ADP donor (e.g., NAD) to an amino acid residue (e.g., threonine) of a substrate (e.g., one or more histone proteins such as H2B). A "mono-ADP-ribosyltransferase" (also referred to herein as mono-ADP ribosylase is a protein (also referred to herein as a polypeptide) which catalyzes the mono-ADP-ribosylation. For example, mono-ADP-ribosyltransferases encompassed by the invention include murine (m) SIR2 genes and proteins (e.g., mSir2 $\alpha$ , mSir2 $\beta$ , mSir2 $\gamma$ ), human Sir2 genes (Frye, *Biochem. Biophys. Res. Commun.* 260:273-279 (1999)) and proteins, yeast genes and proteins that are Homologous to SIR2 (HST) (e.g., HST1, HST2, HST3, HST4), and nucleic acid molecules having accession numbers AI466061, AI465820, or AI465098. (See Figure 19). Database accession numbers for the nucleotide and amino acid sequences for some of these mono-ADP-ribosyltransferases are known. (Frye, *Biochem. Biophys. Res. Commun.* 260:273-279 (1999)). A mSIR2 $\beta$  EST CDNA clone (SEQ ID NO: 34, 35) 557657 can be purchased from Genome Systems, Inc.

Other known mono-ADP-ribosyltransferases include bacterial toxins, such as cholera, pertussis and diphtheria toxins and mammalian glycosylphosphatidylinositol (GP1)-anchored mono-ADP-ribosyltransferases (Domenighini, *et al.*, *Microbiology* 21:667-674 (1996); Moss, *et al.*, *Mol. Cell Biochem.* 193:109-113(1999)). It is understood that any protein that mono-ADP-ribosylates nuclear proteins, in particular histone proteins such as H2B, H2A, or H3, is within the scope of the invention.

A "level of mono-ADP-ribosylation" refers to an amount of a substrate to which an adenosine diphosphate ribose has been transferred. The level of mono-ADP-ribosylation can be assessed using methods known in the art, such as *in vitro* labeling techniques, for example, by monitoring the addition of [ $^{32}$ P]NAD $^{+}$  to a substrate (e.g., H2A, H2B, H3). By way of illustration only, the ADP-ribosylase activity of an agent and the level of mono-ADP-ribosylation of a substrate can be determined by adding a protein to be evaluated (about 0-1 g) to a reaction buffer comprising 50 mM Tris-HCl, pH 8.0, 4 mM MgCl $_2$ , 0.2 mM DTT, 1  $\mu$ M cold or nonradiolabeled NAD, 0.08  $\mu$ M

[<sup>32</sup>P]NAD and admixing or gently vortexing to dilute, resuspend or mix the protein. Substrates (e.g., histone proteins at a concentration of about 0-1g) are then added and the reaction mixture is incubated at ambient temperature (18-25°C) for 30-120 minutes. The presence or absence of ADP-ribosylation products (e.g., ADP-ribosylated nuclear proteins) is detected using autoradiography. Differences in the levels of mono-ADP-ribosylation, for example in the presence and absence of an agent to be tested, can be determined using suitable techniques, including, but not limited to, densitometric scanning of autoradiographs or phosphoimaging techniques of gels. (See, Ausubel, *et al.*, "Current Protocols in Molecular Biology" John Wiley & Sons (1999)). Techniques to perform the mono-ADP-ribosylation assays are detailed in the Exemplification Section.

Confirmation of mono-ADP-ribosylation of substrates and, thus, mono-ADP-ribosyltransferase activity of an agent, can be performed, for example, by adding a suitable amount of snake venom phosphodiesterase (e.g., 2 mg/ml, specific activity 1.5 U/mg) to the resulting product of the reaction mixture described above. The reaction product and phosphodiesterase are incubated at about 37°C for about an hour. Absence of an autoradiographic band following phosphodiesterase digestion, as compared with presence of an autoradiographic band in the absence of digestion, indicates that the substrate was mono-ADP-ribosylated (Figure 4E). Mono-ADP-ribosylase activity can also be verified by the addition of one or more specific mono-ADP-ribosylation inhibitors, including, but not limited to, novobiocin and coumermycin A1, to *in vitro* assays described above (Figure 4F). The inhibitor(s) can be added before or after the addition of the substrate. The absence of a band in an autoradiograph following the addition of a specific mono-ADP-ribosylation inhibitor indicates that the agent has mono-ADP-ribosylase activity. Thus, the mono-ADP-ribosyltransferase activity of agents is evaluated by mono-ADP-ribosylation of substrate proteins as described in the Exemplification Section.

In a preferred embodiment, agonists of the invention are agonists of Sir2 NAD-dependent acetylation of histone proteins and Sir2 mono-ADP-ribosyltransferase

activity. The identification of agonists or antagonists of Sir2 activity can be initially evaluated using yeast cells. In the presence of an agonist of Sir2 activity, a yeast cell will produce a red colonies, whereas antagonists of Sir2 will result in a white colony. When the *ADE2* gene is inserted into one of the three *Sir2*-regulated silencing loci (e.g., telomere rDNA, or HM mating loci) it is silenced in the presence of the agonists, which eventually leads to yeast cells accumulating red pigment. When the *ADE2* gene is transcribed in the presence of antagonists, yeast cells metabolizing the intermediate red pigments and become white in color. Prescreening agents employing color selection of yeast cells will be particularly useful for large scale screening of agents which alter the activity of NAD-acetylation of histone proteins and mono-ADP-ribosyltransferases that ADP-ribosylate nuclear proteins. Following a color selection screening protocol agents can be further evaluated for their ability to alter NAD-dependent acetylation of histone proteins and the level of mono-ADP-ribosylation of nuclear proteins using techniques described herein.

The mono-ADP-ribosyltransferases described herein catalyze the addition of ADP-ribose to cellular proteins, specifically nuclear proteins. A "nuclear protein" refers to any protein, polypeptide or peptide that is located in or performs a function in the nucleus of a eukaryotic cell (e.g., a yeast cell, a zebrafish cell, a *C. elegans* cell, *Drosophila melanogaster* cell, or a mammalian cell, such as a murine cell or a human cell). In a preferred embodiment, the nuclear proteins are histone proteins. Histone proteins are highly conserved DNA-binding nuclear proteins that form the nucleosome, the basic subunit of the chromatin. Histone proteins can be one or more core histone proteins (e.g., H2A, H2B, H3, H4) or an outer histone protein (e.g., H1), or combinations thereof. In a more preferred embodiment, the substrate for mono-ADP-ribosylation is histone H2B or H3.

The term "alters" is defined as a change in an activity (e.g., NAD-dependent acetylation status of at least one amino acid of a histone protein, mono-ADP-ribosylation) or phenomena (e.g., aging, life span), for example, relative to an activity or phenomena in the absence of an agent to be tested. Alteration includes both an increase

and a decrease (e.g., complete abolishment), either qualitatively, quantitatively or both, in the activity or phenomena being monitored.

The agent to be tested (e.g., an agonist or antagonist) can be combined with the cell before or after the addition of the substrate (e.g., a histone protein such as H2B, H3, and/or H4). Experimental conditions for evaluating test substances, such as buffer (e.g., 50 mM Tris-HCl, pH 8.0) or media, concentration (about 0-1 g), temperature (about 18-25°C), and incubation (about 30-120 minutes) requirements, can, initially, be similar to those described in the Exemplification Section herein relating to NAD-dependent acetylation status of histone proteins and mono-ADP-ribosylation of histone proteins by mSir2 $\alpha$  or ySir2. One of ordinary skill in the art can determine empirically how to vary experimental conditions depending upon the biochemical nature of the agent and the particular cell used in the methods described herein.

If the agent to be tested is being administered to an organism, various methods and routes of administration are known in the art. The agent will preferably be formulated in a pharmaceutical composition. For instance, suitable agents can be formulated with a physiologically acceptable medium to prepare a pharmaceutical composition. The particular physiological medium may include, but is not limited to, water, buffered saline, polyols (e.g., glycerol, propylene glycol, liquid polyethylene glycol) and dextrose solutions. The effective amount and optimum concentration of the active ingredient(s) (e.g., the agent) in the chosen medium can be determined empirically, according to procedures well known to medicinal chemists, and will depend on the ultimate pharmaceutical formulation desired. Methods of administration of compositions for use in the invention include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intraocular, oral and intranasal. Other suitable methods of introduction can also include rechargeable or biodegradable devices and slow release polymeric devices. The pharmaceutical compositions of this invention can also be administered as part of a combinatorial therapy with other agents.

The invention further relates to the discovery that increased NAD-dependent deacetylation of histone protein by Sir2 and/or mono-ADP-ribosylation of one or more nuclear proteins (e.g., histone proteins) correlates with decreased rDNA recombination, increased life span and decreased aging at the cellular level. Thus, agents which

5 increase NAD-dependent deacetylation of histone proteins by Sir2 and/or mono-ADP-ribosylation of nuclear proteins are expected to be agents which decrease rDNA recombination, increase life span and decrease aging. Accordingly, in another embodiment, the methods of the invention relate to identifying agents which alter the life span of a cell or an organism. It is envisioned that the methods of the invention can

10 be used for any cell that undergoes aging, for example, a mammalian cell (e.g., from a human), a zebrafish cell, a *C. elegans* cell, or *Drosophila melanogaster* cell in, for example, experimental systems.

The cell or organism is combined with the agent to be tested and the level of NAD-dependent acetylation or deacetylation of histone proteins by Sir2 and/or mono-

15 ADP-ribosylation of one or more nuclear proteins is determined in the presence of the agent and compared to the level in an appropriate control in the absence of the agent. A difference in the level NAD-dependent acetylation of histone proteins and/or of mono-ADP-ribosylation of the nuclear protein in the presence of the agent indicates that the agent alters NAD-dependent acetylation of histone proteins and/or the mono-ADP-

20 ribosylation of a nuclear protein of a cell or an organism and that the agent alters life span of the cell or organism.

Methods of determining life span of a cell or an organism are known in the art. With regard to a yeast cell, for example, "life span" refers to the number of generations, or divisions of a mother cell, which give rise to daughter cells. Techniques to assess the

25 life span of yeast cells, including growth conditions for particular strains, are well known and include stress-resistance methods, cell surface labeling methods and temperature sensitive methods. (See, for example, Guarente *et al.*, U.S. Patent No. 5,874,210 (1999), the teachings of which are incorporated herein in their entirety). The life span of a yeast cell in the presence of an agent can be initially identified, e.g., by



the mean number of generations using a colony color selection strategy. The ability of an agent to alter (e.g., increase) the life span of a cell, as described herein, is measured as an increase in the mean life span of a cell(s) as compared with the mean life span of a cell(s) not combined with the test agent. For example, the life span of the yeast

5 *Saccharomyces cerevisiae* can be increased at least 2-3 fold in the presence of two copies of the yeast or mammalian (e.g., murine, human) NAD-dependent deacetylase and/or mono-ADP-ribosyltransferase SIR2 gene compared to cells lacking the SIR2 gene (Figure 20). When *SIR2* is deleted from yeast haploid cells the life span of cells is reduced by about 50% compared to wild type. When an extra copy of the *SIR2* gene is

10 introduced into wild type yeast haploid cells (the yeast cells have two copies of the gene), the cells display a longer life span than wild type (Kennedy, *et al.*, *Cell* 90:485-496, (1995); Kaeberlein, *et al.*, *Genes & Development*, 13:2370-2580, (1999)).

The life span of mammalian cells (e.g., human and murine diploid fibroblasts, epithelial cells and lymphocytes) refers to the number of population doublings until the

15 cells are in a growth-arrested state such as “cellular senescence” (Hayflick *et al.*, *Experimental Cell Research*, 25:585-621, (1961); Todaro, *et al.*, *Journal of Cell Biology*, 17: 299-313, (1963); Rohme, *Proc. Natl. Acad. Sci. USA*, 78:5009-5013, (1981)). For some mammalian cells which obtain the ability to proliferate indefinitely in culture (e.g., cell lines established from cancers and cell lines engineered chemically

20 or genetically to have infinite life span), the life span can be artificially terminated by the methods of the invention. In this case, the life span is defined as the average doubling cycles before the cells die by the induction of cellular senescence or apoptosis.

The life span of an organism (e.g. vertebrate organism, such as a human, or a nonvertebrate organism, such as an insect, nematode or fish) refers to the average (also

25 referred to herein as mean) duration of life of a given organism. For example, the average life span of a male human being is about 71.8 years, and the average life span of a female human being is about 78.6 years and the average life span of a mouse is about 0.7-2.7 years (Finch, “Longevity, Senescence and the Genoma,” Univ. of Chicago Press, Chicago, ILL (1990)).

In one embodiment, the agent increases the life span of the cell or organism by increasing the NAD-dependent deacetylation of histone proteins, in particular at least one amino acid at the N-terminal lysine residues of H3 (e.g., lysine 9 and/or lysine 14) and/or H4 (e.g., lysine 16), by increasing the activity of Sir2. In another embodiment, the agent increases the lifespan of the cell or organism by increasing the level of mono-ADP-ribosylation of a nuclear protein, such as H2B or H3, in particular mono-ADP-ribosylation of a lysine residues in H3 and/or H4. An increase in life span would be a duration of a particular life beyond the average for that cell or organism.

In another embodiment, the agent decreases the life span of the cell or organism by decreasing the activity of Sir2, thereby altering NAD-dependent acetylation (e.g., deacetylation) of histone proteins, in particular at least one amino acid at the N-terminus of H3 (e.g., lysine 9 and/or lysine 14) and/or H4 (e.g., lysine 16). In an additional embodiment, the agent decreases the level of mono-ADP-ribosylation of a nuclear protein, such as H2B or H3. A decrease in life span would a duration of a particular life less than average for that cell organism.

The invention also pertains to a method of identifying an agent which alters aging of a cell or an organism. The cell or organism and agent to be tested are combined and the level of NAD-dependent acetylation of histone proteins and/or the level of mono-ADP-ribosylation of a nuclear protein (e.g., H2B, H2A or H3) is determined and compared to the NAD-dependent acetylation status of a histone protein and/or the level of mono-ADP-ribosylation of the nuclear protein in the absence of the agent. A difference in the NAD-dependent acetylation status and/or level of mono-ADP-ribosylation in the presence and absence of an agent indicates that the agent alters aging of the cell or organism.

“Aging” refers to all time-dependent changes to which biological entities, from molecules to ecosystems, are subject, though the mechanisms and consequences to function may be vastly different (Medawar, “An Unsolved Problem of Biology”, H.K. Lewis, London, (1952). Additionally or alternatively, aging refers to the process of growing old or senescing. Aging is associated with particular phenotypes in a cell or

organism. In a cell, aging can be characterized by an increase in cell size, a slowing of the cell cycle, shortening of telomeres, and/or expression of some particular categories of genes (Stanulis-Praeger, *Mech. Ageing Dev.* 38:1-48, (1987); Faraher, *et al.*, *BioEssay*, 20:985-991, (1998)). The changes in heterochromatin structure and mitochondria can also be associated with aging (Imai, *et al.*, *Experimental Gerontology*, 33:555-570, (1998); Wallace, *et al.*, *Biofactors*, 7:187-190, (1998)). In yeast cells, sterility, the appearance of surface wrinkles, blebs and bud scars, nucleolar enlargement and fragmentation, and formation, replication, and accumulation of rDNA circles are also associated with aging (Sinclair, Mills and Guarente, *Annu. Rev. Microbiol.*, 52:533-560, (1998)).

In yeast, aging is defined by the relatively fixed number of cell divisions undergone by mother cells (Müller *et al.*, *Aging Dev.* 12:47 (1980)), as well as characteristic changes during their life span (Jazwinski, *Science* 273:54 (1996)), such as cell enlargement (Mortimer and Johnson, *Nature* 183:1751 (1959)) and sterility (Müller, *J. Microbiol. Serol.* 51:1 (1985)). Techniques to determine aging in a yeast cell are well-known and are described, for example, in Guarente *et al.*, U.S. Patent No. 5,874,210 (1999), Smeal, *et al.*, *Cell* 84:633-642 (1996); Sinclair, *et al.*, *Science* 277:1313-1316 (1997); Sinclair, *et al.*, *Cell* 91:1033-1042 (1997)), the teachings of all of which are incorporated herein in their entirety.

Aging of an organism refers to the deterioration of the organism associated with changes in structures and functions (e.g., phenotype) that are characteristic of a particular organism. Such characteristics can include, for example, hair loss, graying of hair, osteoporosis, cataracts, atherosclerosis, loss of skin elasticity and a propensity for certain cancers.

The agents identified by the methods of the present invention can be used to alter the aging of a cell or an organism by altering the level Sir2 activity, NAD-dependent acetylation status of histone proteins and/or of mono-ADP-ribosylation of a nuclear protein. In a preferred embodiment, the agents identified by the methods of the

invention decrease aging of the cell or organism. In another embodiment, the agents identified by the methods of the invention increase aging of the cell or organism.

Due to their ease of experimental manipulation, similar structural features associated with aging in mammalian cells (Guarente *et al.*, U.S. Patent No. 5,874,210 (1999)), and an Sir2 protein which shares sequence (nucleic acid and amino acid) and enzymatic (e.g., NAD-dependent acetylation of histone proteins, mono-ADP-ribosyltransferase) with a mammalian Sir2 proteins (e.g., mSir2 $\alpha$ , mSir2 $\beta$ ), the yeast cell is a suitable cellular model to identify agents which alter NAD-dependent acetylation of histone proteins or mono-ADP-ribosylation of nuclear proteins, increase life span and decrease aging in mammalian cells and organisms as well as nonmammalian cells and organisms (e.g., insect, nematode, fish). Moreover, a mammalian (e.g., mSir2 $\alpha$ ) NAD-dependent deacetylase and/or mono-ADP-ribosylase increases the life span and slows aging of yeast cells. Thus, mammalian and yeast Sir2 proteins mediate similar biological activities.

As defined herein, mammals include rodents (such as rats, mice or guinea pigs), domesticated animals (such as dogs or cats), ruminant animals (such as horses, cows) and primates (such as monkeys or humans).

Thus, the agents identified by the methods described herein, which increase life span or decrease aging as described above, can be used to increase the life span or decrease aging of cells, e.g., mammalian cells including human cells, or vertebrate (e.g., mice, humans, guinea pigs, rats) or invertebrate (e.g., insects, nematodes) organisms. For example, cells to be treated can be combined with an effective amount of an agent that increases the activity of Sir2, increases the NAD-dependent deacetylation of histone proteins, and/or level of mono-ADP-ribosylation of a nuclear protein, thereby increasing their life span or decreasing their aging relative to untreated cells or organisms.

An "effective amount" of an agent, as used herein, is defined as that quantity of an agent which increases life span or decreases aging, to any degree, in the cell or organism being treated by increasing, to any degree, the NAD-dependent deacetylation of histone proteins by Sir2 or a Sir2-like protein and/or the level of mono-ADP-

ribosylation of a nuclear protein (e.g., H2B, H2A, H3, or H4). In a preferred embodiment, lysines at position 9 and/or 14 in H3 are deacetylated and/or lysine at position 16 in H4 is deacetylated. For example, an effective amount of an agonist of a mono-ADP-ribosyltransferase in a cell can inhibit certain characteristics associated with aging, for example, the formation of rDNA with, or without, inhibiting other characteristics, such as the replication and/or accumulation of rDNA. Similarly, an effective amount of an agent that increases the NAD-dependent deacetylation of histone proteins (e.g., H2B, H3 and/or H4) and/or the levels of mono-ADP-ribosylation of a nuclear protein can decrease aging in an organism by slowing hair loss, with or without, diminishing graying or hair, cataracts or atherosclerosis.

It is envisioned that the agents identified by the methods of the present invention can be administered to cells or organisms which age naturally (e.g., normally) or age prematurely or abnormally (e.g., as a result of Werner's syndrome in mice or humans) due to genetic permutations. Thus, the agents identified by the methods of the present invention, can be useful in anti-aging therapies and for the treatment of aging diseases, such as Werner's syndrome, to prevent, halt, hasten, slow or ameliorate premature aging. Additionally, or alternatively, the identification of agents which alter the NAD-dependent acetylation status of histone proteins (e.g., lysines 9 and/or 14 of H3, lysine 16 of H4) and/or the mono-ADP-ribosylation of nuclear proteins can be also be useful for the continued study of cellular and organismal aging processes.

Thus, the present invention also pertains to methods of increasing the life span or decreasing the aging of a cell or an organism by administering an effective amount of an agent that increases the NAD-dependent deacetylation of histone proteins and/or increases the mono-ADP-ribosylation of a nuclear protein. The agent is identified by combining an agent to be tested with a cell or an organism and determining the level of NAD-dependent deacetylation of histone proteins (e.g., H3, H4) and/or mono-ADP-ribosylation of a nuclear protein in the presence of the agent which is compared to the NAD-dependent acetylation of a histone protein and/or level of mono-ADP-ribosylation of a nuclear protein in the absence of the agent. The presence of an agent which

increases life span or decreases aging increases the activity of Sir2 thereby increasing the deacetylation of histone proteins and/or increases the level mono-ADP-ribosylated nuclear protein (e.g., H2B, H2A, H3, H4).

The present invention also pertains to methods of increasing the life span or  
5 decreasing aging of a cell or an organism by administering to the cell or organism an agonist of Sir2 that increases the NAD-dependent deacetylation of histone proteins, a mono-ADP-ribosyltransferase (e.g., Sir2) or an agonist of a mono-ADP-ribosyltransferase of a nuclear protein. In a preferred embodiment, the nuclear protein is a histone protein, in particular H2A, H2B, H3 or H4. In a more preferred embodiment,  
10 the acetylation status of lysine residues at positions 9 and/or 14 of H3 are altered and the lysine at position 16 of H4 is altered by altering the activity of Sir2. Preferably the mono-ADP-ribosyltransferase is a Sir2 protein (e.g., Sir2 $\alpha$ , Sir2 $\beta$ , Sir2 $\gamma$ ). Specifically, the core domain of a Sir2 protein (Figure 2B, 6A, 14a, 19; SEQ ID NOS: 2, 3, 4, 5, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 and 24) alters the NAD-dependent  
15 acetylation status of histone proteins, the mono-ADP-ribosyltransferase or the agonist of NAD-dependent acetylation status of histone proteins and/or mono-ADP-ribosyltransferase activity of the core domain of Sir2.

Because aging in cells is associated with the formation, replication and/or accumulation of ribosomal DNA (rDNA) circles, the present invention relates to  
20 methods of inhibiting the formation, replication and/or accumulation of rDNA circles in a cell or an organism by administering an effective amount of a mono-ADP-ribosyltransferase or agonist of NAD-dependent acetylation status of histone proteins and/or a mono-ADP-ribosyltransferase to a cell or organism. rDNA circles accumulate exponentially in old yeast mother cells and are responsible for age-related enlargement  
25 and fragmentation of the nucleolus, and appear to nucleate the fragmented nucleolus. (Sinclair, *et al.*, *Cell* 91:1033-1042(1997)). For example, agents which have been identified by methods described herein as agonists of NAD-dependent deacetylation and/or of mono-ADP-ribosylation of nuclear proteins can be administered to a cell or an organism to suppress the formation, replication and/or accumulation of rDNA circles.

The cell samples thus treated are then examined for longer-lived, slower aged colonies, using any of the methods described herein or other appropriate methods.

The invention further relates to a method of suppressing recombination between rDNA in a cell or an organism comprising administering to the cell or the organism a  
5 mono-ADP-ribosyltransferase or an agonist of NAD-dependent deacetylation of histone proteins and/or a mono-ADP-ribosyltransferase. Recombination between rDNA is a characteristic of aging in cells. In yeast cells, sterility associated aging is due to a loss of transcriptional silencing at *HMRa* and *HMLα* loci, and the resulting expression of both *a* and *α* mating-type information (Smeal *et al.*, *Cell* 84:633 (1996)). The Sir2  
10 protein silences genes inserted at rDNA (Bryk *et al.*, *Genes Dev.* 11:255 (1997); Smith and Boeke, *Genes Dev.* 11:241 (1997)) and suppresses recombination between rDNA repeats (Gottlieb and Esposito, *Cell* 56:771 (1989)). Thus, suppressing recombination between rDNA by administering a mono-ADP-ribosyltransferase or an agonist of a NAD-dependent deacetylation of histone proteins and/or mono-ADP-ribosyltransferase  
15 can also decrease aging and increase life span.

Agents which alter the NAD-dependent acetylation status of histone proteins and/or mono-ADP-ribosylation of nuclear proteins (e.g., agonist of NAD-dependent deacetylation and/or mono-ADP-ribosyltransferase activity) can be formulated into compositions. Such compositions can also comprise a pharmaceutically acceptable  
20 carrier, and are referred to herein as pharmaceutical compositions. The agonist or antagonist compositions of the present invention can be administered intravenously, parenterally, orally, nasally, by inhalation, by implant, by injection, or by suppository. The agonist or antagonist compositions can be administered in a single dose or in more than one dose over a period of time to achieve a level of NAD-dependent deacetylation  
25 of histone proteins and/or mono-ADP-ribosylation of nuclear protein which is sufficient to confer the desired effect.

Suitable pharmaceutical carriers include, but are not limited to sterile water, salt solutions (such as Ringer's solution), alcohols, polyethylene glycols, gelatin, carbohydrates such as lactose, amylose or starch, magnesium stearate, talc, silicic acid,

viscous paraffin, fatty acid esters, hydroxymethylcellulose, polyvinyl pyrrolidone, etc. The pharmaceutical preparations can be sterilized and if desired, mixed with auxiliary substances, e.g., lubricants, preservatives, stabilizers, wetting substances, emulsifiers, salts for influencing osmotic pressure, buffers, coloring, and/or aromatic substances and  
5 the like which do not deleteriously react with the agonist or antagonist compounds. They can also be combined where desired with other active substances, e.g., enzyme inhibitors, to reduce metabolic degradation.

For parenteral application, particularly suitable are injectable, sterile solutions, preferably oily or aqueous solutions, as well as suspensions, emulsions, or implants,  
10 including suppositories. Ampules are convenient unit dosages.

It will be appreciated that the actual effective amounts of an agonist or antagonist of NAD-dependent acetylation status of histone proteins and/or mono-ADP-ribosylation of nuclear proteins in a specific case can vary according to the specific agonist or antagonist being utilized, the particular composition formulated, the mode of  
15 administration and the age, weight and condition of the patient, for example. For example, an effective amount of an agonist is an amount of agonist capable of slowing aging or extending the life span of a cell or an organism by altering the NAD-dependent acetylation status and/or mono-ADP-ribosylating substrates such as nuclear proteins (e.g., histone proteins, preferably H2A, H2B, H3). Dosages of suitable agonists or  
20 antagonists of NAD-dependent deacetylation of histone proteins and/or mono-ADP-ribosylation of nuclear proteins for a particular patient can be determined by one of ordinary skill in the art using conventional considerations, (e.g. by means of an appropriate, conventional pharmacological protocol).

The present invention further relates to novel murine SIR2 (Silent Information  
25 Regulator) genes (e.g., mSIR2 $\alpha$ , mSIR2 $\beta$ ) and their protein products. The invention also relates to the discovery that mSir2 $\alpha$  and yeast Sir2 (ySir2) proteins possess mono-ADP-ribosyltransferase activity, in particular mSir2 $\alpha$  and ySir2 mono-ADP-ribosylate histone proteins (e.g., H2B, H3, H4). As shown in the Exemplification Section, transfection of yeast cells, which lack SIR2, with ySIR2 genes, slows aging and extends



the life span of cell. Specifically, the administration of two copies of the ySIR2 gene increases life expectancy of the cell about 1.5 fold over cells with a single copy of the SIR2 gene and about 2-3 fold above cells which do not contain the SIR2 gene. Thus, the SIR2 gene of the present invention plays a critical role in the life span and aging of a cell and, thus, of a multicellular organism such as a mammal.

The terms "SIR2" and "Sir2 amino acid sequence or Sir2 protein" refer to the SIR2 gene and its protein product, respectively. SIR2 or Sir2 refers to the any of the yeast (e.g., *S. cerevisiae*) SIR2 or Sir 2. "mSIR2" or "mSir2" refers to any murine SIR2 (e.g., SIR2 $\alpha$ , SIR2 $\beta$ , SIR2 $\gamma$ ). The terms "SIR2 nucleic acid", "nucleotide sequence of SIR2" or the "corresponding SIR2 nucleic acid", refer to the nucleic acid sequence that encodes the Sir2 protein.

The present invention also relates to the nucleic acid sequences encoding murine Sir2 proteins (e.g., mSir2 $\alpha$ , mSir2 $\beta$ , mSir2 $\gamma$ ). In particular a full length cDNA and genomic DNA nucleic acid sequence of mSIR2 $\alpha$  and a partial cDNA sequence of SIR2 $\beta$ . The murine Sir2 proteins (e.g., mSir2 $\alpha$ , mSir2 $\beta$ , mSir2 $\gamma$ ) are encoded by at least three different genes. The nucleic acid sequence (SEQ ID NO.: 25) that encodes mSir2 $\alpha$  protein (SEQ ID NO.: 26) can be found in Figure 21.

The Sir2 proteins have functional domains. For example, one domain can be the core domain and another domain can be the amino-terminus or carboxy-terminus. The core-domain of the Sir2 protein (e.g., mSir2 $\alpha$ ) can alter the NAD-dependent acetylation status of histone proteins and/or mono-ADP-ribosyltransferase activity. The invention is also intended to embody the functional domains (e.g., core-domain of Sir2), as described herein. The amino acid sequence of the core domain of the mSir2 $\alpha$  protein (SEQ ID NOS.: 4, 9, 12 and 19) is depicted in Figures 2C, 6, 14a and 19. Amino acid sequences (SEQ ID NOS: 2, 3, 5, 10, 11, 13, 14, 15, 16, 17, 18, 20, 21, 22, 23 and 24) which share structural identity to the nucleic acid and amino acid sequences of mSir2 $\alpha$  are depicted in Figures 2C, 6A, 14a and 19.

The term "isolated", as it refers to a Sir2 protein, means a protein, as found in nature, but separated away from other proteins and cellular material of their source of origin. The isolated Sir2 proteins include essentially pure protein from cells, proteins produced by chemical synthesis, by combinations of biological and chemical synthesis and/or by recombinant methods.

The invention is intended to encompass Sir2 proteins, and proteins and polypeptides having amino acid sequences analogous (also referred to herein as similar or homologous) to the amino acid sequences of the Sir2 protein (e.g., mSir2 $\alpha$ , ySir2) and functional equivalents or fragments thereof. Such proteins are defined herein as Sir2-like proteins, Sir2 homologs (e.g., analogues) or derivatives. Any protein possessing the Sir2 consensus sequence GAGISTS(L/A)GIPDFR (SEQ ID NO: 27) or YTQNID (SEQ ID NO: 28) (Brachmann, *et al.*, *Genes & Development* 9:2888-2902 (1995) and which is capable of NAD-dependent deacetylation and/or mono-ADP-ribosylation of nuclear proteins is within the scope of the invention.

It is also envisioned that other Sir proteins (e.g., Sir1, Sir3, Sir4) or Sir-like proteins and the nucleic acids (e.g., SIR1, SIR3, SIR4) which encode Sir proteins, other than Sir2 or Sir2-like proteins, are within the scope of the invention.

Analogous or homologous amino acid sequences are further defined herein to mean amino acid sequences with sufficient identity to the Sir2 amino acid sequences so as to possess the biological activity of a Sir2 protein (e.g., mono-ADP-ribosylation of histone proteins). For example, an analogous peptide can be produced with "silent" changes in amino acid sequence wherein one, or more, amino acid residues differ from the amino acid residues of the Sir2 protein, yet still possess the biological activity of Sir2. Examples of such differences include additions, deletions or substitutions of residues of the amino acid sequence of Sir2. Also encompassed by the invention are analogous proteins that exhibit greater, or lesser, NAD-dependent deacetylation of histone proteins and/or mono-ADP-ribosyltransferase activity (e.g., biological activity) of the Sir2 protein of the present invention. Such structurally and functionally related proteins are also referred to herein as "Sir2-like" proteins.

The "biological activity" of a Sir2 protein is defined herein to mean the ability to alter the NAD-dependent acetylation status of a histone protein and/or the mono-ADP-ribosylates a substrate such as a histone protein (e.g., H2B, H2A, H3, H4). In particular, the biological activity of mSir2 $\alpha$  is the ability to alter the NAD-dependent acetylation status of lysine residues in the N-terminus of H3 (e.g., lysine 9 and/or 14) and/or H4 (e.g., lysine 16) and/or mono-ADP-ribosylate an amino acid residue of H2B, H3 or H4. Additionally, or alternatively, the biological activity also means the ability to slow aging or extend life span by, for example, repressing recombination of rDNA, or inhibiting the formation, replication and/or accumulation of rDNA circles of a cell or an organism. The biological function of Sir2 is illustrated and further defined by the Exemplification Section.

The invention also encompasses biologically active polypeptide fragments of the Sir2 protein, as described herein. Such fragments can include only a part of a full length amino acid sequence of Sir2 and yet possess the ability to NAD-dependent deacetylation and/or mono-ADP-ribosylate a substrate. Specifically encompassed by the invention are fragments of Sir2 comprising the core domain of Sir2.

The term "core domain" (also referred to herein as "core") refers to the evolutionarily conserved domains for Sir2 or Sir2-like proteins which can be identified, for example, by the comparison of amino acid sequences by, for example, CLUSTAL X, BLAST, PSI-BLAST or FASTA algorithms. The "core domain" is the domain that shows significant identity and/or homology to about 240-270 amino acids of Sir2 or Sir2-like proteins (about 20-50% or higher as amino acid identity, see Figure 2) and/or possesses the consensus sequence GAG(V/I)S(T/V)S(L/C/A)GIPDFRS (SEQ ID NO: 27) and YTQNID (SEQ ID NO: 28) (Brachmann, *et al.*, *Genes & Development* 9:2888-2902, (1995)). The "core domain" of Sir2 proteins has NAD-dependent deacetylation and/or mono-ADP-ribosylation activities. Any protein with a "core domain" of a Sir2 protein, a fragment of the core domain, or any functional or structural equivalent which is capable of NAD-dependent deacetylation and/or mono-ADP-ribosylation of nuclear proteins is within the scope of the invention.

The Sir2 protein and nucleic acid sequence include homologues, as defined herein. The homologous proteins and nucleic acid sequences can be determined using methods known to those of skill in the art. Initial homology searches can be performed at NCBI against the GenBank (release 87.0), EMBL (release 39.0), and SwissProt (release 30.0) databases using the BLAST and PSI-BLAST network services. (See, for example, Altshul, *et al.*, *J. Mol. Biol.* 215: 403 (1990); Altschul *et al.* *Nucleic Acid Res.* 25: 3389-3402 (1997); Altschul, *Nucleic Acids Res.* 25:3389-3402 (1998), the teachings of which are incorporated herein by reference). Computer analysis of nucleotide sequences can be performed using the MOTIFS and the FindPatterns subroutines of the Genetics Computing Group (GCG, version 8.0) software. Protein and/or nucleotide comparisons can also be performed according to CLUSTAL algorithms (Higgins, *et al.*, *Gene*, 73: 237-244 (1988)). Homologous proteins and/or nucleic acid sequences to the Sir2 protein and/or nucleic acid sequences that encode the Sir2 protein are defined as those molecules with greater than 25% sequences identity, also referred to herein as similarity, (e.g., 30%, 40%, 50%, 60%, 70%, 80% or 90% homology). The percent identity between the yeast (*S. cerevisiae*) SIR2 sequence and the murine SIR2 $\alpha$  and SIR2 $\beta$  sequence is 45.9% (Figure 2D). These particular sequence similarities and identities were determined using the CLUSTAL algorithm.

Homology was determined in accordance with the methods described in above (e.g., BLAST, PSI-BLAST algorithms). Homologs of mSIR2 $\alpha$  can be, for example, mSIR $\gamma$ , mSIR2 $\beta$ , ySIR2, yHST1, yHST2, yHST3, yHST4 (amino acid sequences depicted in SEQ ID NOS: 2, 3, 5, 10, 11, 13, 14, 15, 16, 17, 18, 20, 21, 22, 23 and 24; Figure 2C, 6A, 14a and 19) and human SIR2 nucleic acid or protein (Frye, *Biochem. Biophys. Res. Commun.* 260:273-279 (1999)).

Sequence identity of homologs of SIR2 can be to the entire nucleic acid or amino acid sequence, or to the core domain of the protein. In one embodiment, the BLAST or PSI-BLAST parameters are set such that they yield a sequence having at least about 60% sequence identity with the corresponding known mSIR2 $\alpha$  sequence, preferably, at least about 70% sequence. In another embodiment, the percent sequence

identity is at least about 85%, and in yet another embodiment, at least about 95%. Such molecules are also referred to herein as Sir2-like proteins. Thus, the Sir2-like proteins possess structural identity with the SIR2 molecules (e.g., nucleic acid or amino acid identity) described herein. The Sir2-like proteins are capable of altering the NAD-  
5 dependent acetylation status of at least one amino acid in a histone protein (e.g., lysine 9 and/or 14 of H3, lysine 16 of H4) and/or mono-ADP-ribosylating substrates (e.g., histone proteins), thus extending the life span or slowing the aging of a cell or an organism.

Biologically active derivatives or analogs of the Sir2 protein can be made using  
10 peptide mimetics, designed and produced by techniques known to those of skill in the art. (see e.g., U.S. Patent Nos. 4,612,132; 5,643,873 and 5,654,276, the teachings of which are incorporated herein by reference). These peptide mimetics can be based, for example, on a specific Sir2 amino acid sequence such as the core domain of Sir2 $\alpha$ . These peptide mimetics can possess biological activity similar to the biological activity  
15 of the corresponding peptide compound, but possess a "biological advantage" over the corresponding Sir2 amino acid sequence with respect to one, or more, of the following properties: solubility, stability and susceptibility to hydrolysis and proteolysis.

It is also envisioned that any NAD-dependent deacetylase that alters the acetylation status of at least one amino acid of a histone protein and/or a mono-ADP-  
20 ribosyltransferase or Sir2-like protein containing proteins functionally equivalent to the Sir2 proteins described herein will be within the scope of the invention. The phrase "functionally equivalent" as used herein refers to any molecule (e.g., peptide, peptide mimetic, protein and nucleic acid sequence encoding the protein) which mimics the NAD-dependent deacetylation activity of Sir2 and/or mono-ADP-ribosyltransferase  
25 activity of the Sir2 $\alpha$  proteins and/or functional domains of Sir2 (e.g., the carboxy-terminus) described herein (such as Sir2 $\beta$ , Sir2 $\gamma$ , HST1, HST2, HST3) or which exhibit nucleotide or amino acid sequence identity to Sir2 $\alpha$  such as Sir2 $\beta$ , Sir2 $\gamma$ , for example.

The Sir2 protein or Sir2-like protein can also be mutated to down-regulate or affect one or more properties of the protein, while other properties still exist. In

particular, the Sir2 protein can be mutated at a position in the sequence that prevents it from catalyzing the transfer of an ADP ribose from a donor, such as NAD, to a substrate and/or from acetylating or deacetylating at least one amino acid of a histone protein (e.g., lysine 9 and/or 14 of H3, lysine 16 of H4).

- 5           Thus, another embodiment of the present invention encompasses mutants of Sir2 proteins with altered NAD-dependent deacetylation activity and/or mono-ADP-ribosyltransferase activity. Mutants with NAD-dependent deacetylation activity and/or mono-ADP-ribosyltransferase activity greater than that of wildtype (e.g., nonmutant, naturally occurring) Sir2 are referred to as having "increased" NAD-dependent
- 10   deacetylation activity or "increased" mono-ADP-ribosyltransferase activity. Mutants with a mono-ADP-ribosyltransferase activity less than that of wildtype Sir2 are referred to as having "decreased" NAD-dependent deacetylation activity or "decreased" mono-ADP-ribosyltransferase activity. The mutants of the present invention can be used, for example, to further understand the mechanism of substrate specificity and cellular
- 15   pathways influenced by NAD-dependent deacetylation of histone proteins and/or mono-ADP-ribosylation of substrates, such as histone proteins.

- The term "mutant", as used herein, refers to any modified nucleic acid sequence encoding a Sir2 protein or Sir2-like protein. For example, the mutant can be a protein produced as a result of a point mutation or the addition, deletion, insertion and/or
- 20   substitution of one or more nucleotides encoding the Sir2 protein, or any combination thereof. Modifications can be, for example, conserved or non-conserved, natural or unnatural.

- As used herein a mutant also refers to the protein encoded by the mutated SIR2 nucleic acid. That is, the term "mutant" also refers to a protein (also referred to herein
- 25   as polypeptide) which is modified at one, or more, amino acid residues from the wildtype (e.g., naturally occurring) Sir2 protein. In a preferred embodiment, mutants are generated by mutations in mSir2 $\alpha$  or ySir2 proteins. In a particular embodiment, the core domain of the mSir2 $\alpha$  protein, as described herein, has a mutation resulting in a altered mono-ADP-ribosyltransferase activity. For example, in this embodiment the

mSir2 $\alpha$  mutant is a mutant of mSir2 $\alpha$  resulting from a point mutation substituting the glycine at position 253 in the core domain of mSir2 $\alpha$  with alanine residue to generate the G253A Sir2 $\alpha$  mutant. Sir2 mutants can be made by mutations to one, or more, amino acid residues selected from a group consisting of glycine, serine, proline, isoleucine, phenylalanine, threonine, or histidine, or any combination thereof.

As used herein the designation for the amino acid substitutions for the Sir2 mutants is indicated as, for example, "G253A", wherein the letter to the left of the number indicates the amino acid in the wildtype Sir2 protein (e.g., "G" or glycine); the number indicates the position of the amino acid in the wildtype Sir2 protein (e.g., position 253); and the letter to the right of the number indicates the amino acid residue which replaces the wildtype (e.g., "A" or alanine).

Additionally, or alternatively, the mutants are designated by the amino acid which is mutated to an alanine residue, for example Thr-261, Gly-270, Iso-271, Arg-275 or Asn-345. Specifically encompassed by the invention are mSir2 $\alpha$  mutants G253A, G255A, S257A, I262A, F265A, R266A, G270A (also referred to herein as Gly-270), P285A, T336A, H355A; and ySir2 mutants Thr-261, Iso-271, Arg-275, Asn-345 and Asp-347.

Using well-known techniques to align amino acids, amino acid residues suitable for mutation as described herein for mSir2 $\alpha$  or ySir2 proteins can be determined for other Sir2 (e.g., Sir2 $\beta$ , Sir2 $\gamma$ ) or Sir2-like (e.g., HST1, HST2) proteins. Nucleic acid sequences encoding the Sir2 proteins can be mutated; the mutated nucleic acid constructs expressed under standard experimental conditions well known to the skilled artisan; and the resulting mutant proteins evaluated for NAD-dependent deacetylation of histone proteins and/or mono-ADP-ribosyltransferase activity, the ability to slow aging, or extend life span as described herein.

It is also envisioned that fragments of the Sir2 proteins can be used in the methods of the invention. "Fragments" of Sir2 proteins, as used herein, refer to any part of the Sir2 protein capable of altering the NAD-dependent acetylation status of at least one amino acid residue of a histone protein (e.g., lysine 9 and/or 14 of H3, lysine 16 of

H4) and/or mono-ADP-ribosylating a substrate (e.g., H2B, H3, or H4). For example, the core domain of the Sir2 protein is considered a fragment of Sir2.

A Sir2 protein can be in the form of a conjugate or a fusion protein which can be manufactured by known methods. Fusion proteins can be manufactured according to known methods of recombinant DNA technology. For example, fusion proteins can be expressed from a nucleic acid molecule comprising sequences which code for a biologically active portion of the Sir2 protein and a fusion partner, for example, a portion of an immunoglobulin or a peptide linker. A nucleic acid construct encoding a Sir2 fusion protein can be introduced into a host cell, expressed, isolated or purified from a cell by means of an affinity matrix employing antibodies to Sir2 or the fusion partner (e.g., IgG).

Antibodies can be raised to the Sir2 protein, Sir2-like protein, analogs, and portions thereof, using techniques known to those of skill in the art. (See, for example, Kohler *et al.*, *Nature* 256:495-497 (1975); Cole *et al.*, "Monoclonal Antibodies and Cancer Therapy", Alan R. Liss, Inc., pp. 77-96 (1985); Ausubel *et al.*, "Current Protocols in Molecular Biology", John Wiley & Sons, Inc. (1999)). A mammal, such as a murine, rat, hamster or rabbit, can be immunized with an immunogenic form of the protein (e.g., mSir2 $\alpha$ , mSir2 $\beta$ , or a peptide comprising an antigenic fragment of the protein which is capable of eliciting an antibody response). Techniques for conferring immunogenicity on a protein or peptide include conjugation to carriers, such as BSA or keyhole limpet hemocyanin, or other techniques, such as the use of Freud's adjuvant or Titermax, well known in the art. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA, RIA or Western blots can be used with the Sir2 protein to assess the levels of antibody.

In a preferred embodiment, the antibody, or antigen-binding fragment, selectively binds murine Sir2 proteins, such as mSir2 $\alpha$  and mSir2 $\beta$ . An "antigen-binding fragment" of an antibody refers to an antibody which binds to any part of the Sir2 protein.



The antibodies of the invention can be human and nonhuman antibodies. The antibodies can be polyclonal, monoclonal, chimeric (e.g., human chimeric antibodies), or fragments thereof. These antibodies can be used to purify or identify the Sir2 protein contained in a mixture of proteins, using techniques well known to those of skill in the art. These antibodies, or antigen-binding fragments thereof, can be used to detect the presence or absence of the Sir2 protein using standard immunochemistry, Western blot methods, ELISA, or RIA. For example, and ELISA and RIA assays can be used to quantitate the amount of Sir2 in a cellular or nuclear extract.

As used herein, an "isolated" nucleic acid molecule (e.g., gene, nucleotide sequence, nucleic acid sequence), when referring to an Sir2 protein encoded by a nucleic acid sequence, is a nucleic acid molecule which is not flanked by nucleic acid molecules which normally (e.g., naturally occurring) flank the gene or nucleotide sequence (e.g., as in genomic sequences) and/or has been completely or partially purified from other transcribed sequences (e.g., as in a cDNA library). Thus, an isolated nucleic acid molecule, gene or nucleotide sequence can include a nucleic acid molecule, gene or nucleotide sequence which is synthesized chemically or by recombinant means. Nucleic acid molecules contained in a vector are included in the definition of "isolated" as used herein. Also, isolated nucleic acid molecules include recombinant nucleic acid molecules and heterologous host cells, as well as partially or substantially or purified nucleic acid molecules in solution. *In vivo* and *in vitro* RNA transcripts of the invention are also encompassed by "isolated" nucleic acid molecules. Such isolated nucleic acid molecules are useful for introduction into host cells, the manufacture of the encoded Sir2 protein, as probes for isolating homologues sequences (e.g., from other mammalian species or other organisms), for gene mapping (e.g., by *in situ* hybridization), or for detecting the presence (e.g., by Southern blot analysis) or expression (e.g., by Northern blot analysis, RNase protection assays) of related SIR2 genes in cells or tissue.

The present invention also encompasses isolated nucleic acid sequences encoding the Sir2 proteins described herein, and fragments of nucleic acid sequences encoding biologically active Sir2 proteins. Fragments of the nucleic acid sequences,

described herein, as useful as probes to detect the presence of SIR2. Specifically provided for in the present invention are DNA/RNA (also referred to herein as nucleic acid) sequences encoding mSir2 $\alpha$  and mSir2 $\beta$  proteins, the fully complementary strands of these sequences, and allelic variations thereof. Also encompassed by the present invention are nucleic acid sequences, genomic DNA, cDNA, RNA or a combination thereof, which are substantially complementary to the nucleic acid sequences (e.g., DNA, RNA) encoding Sir2 (e.g., mSir2 $\alpha$ , mSir2 $\beta$ ), and which specifically hybridize with the SIR2 nucleic acid sequences under conditions of stringency known to those of skill in the art, those conditions being sufficient to identify nucleic acid sequences with substantial nucleic acid identity. As defined herein, substantially complementary means that the sequence need not reflect the exact sequence of the SIR2 nucleic acid, but must be sufficiently similar in identity of sequence to hybridize with SIR2 nucleic acid under stringent conditions. Conditions of stringency are described, for example, in Ausubel, *et al.*, "Current Protocols in Molecular Biology", John Wiley & Sons, Inc. (1999). Non-complementary bases can be interspersed in the sequence, or the sequences can be longer or shorter than SIR2 nucleic acid, provided that the sequence has a sufficient number of bases complementary to SIR2 to hybridize therewith.

The SIR2 nucleic sequence, or a fragment thereof, can be used as a probe to isolate additional SIR2 homologs. For example, a cDNA or genomic DNA library from the appropriate organism can be screened with labeled SIR2 nucleic acid probes (e.g., DNA or RNA) to identify homologous genes, as described, for example, in Ausubel, F.M., *et al.*, "Current Protocols in Molecular Biology", John Wiley & Sons, (1999).

Typically the nucleic acid probe comprises a nucleic acid sequence of sufficient length and complementary to specifically hybridize to nucleic acid sequences which encode mSir2 $\alpha$  (SEQ ID NO: 25), mSir2 $\beta$ , mSir2 $\gamma$ , ySir2 or human Sir2. The requirements of sufficient length and complementarity can be easily determined by one of skill in the art.

Uses of nucleic acids encoding cloned Sir2 proteins or fragments include one or more the following: (1) producing proteins which can be used, for example, for structure

determination, to further assess Sir2 NAD-dependent deacetylation activity and/or mono-ADP-ribosyltransferase activity, or to obtain antibodies binding to the Sir2 protein; (2) to determine the nucleotide sequence encoding a Sir2-like protein which can be used, for example, as a basis for comparison with other proteins to determine

5 conserved regions, determine unique nucleotide sequences for normal and altered proteins, and to determine nucleotide sequences to be used as target sites for antisense nucleic acids, ribozymes, hybridization detection probes, or PCR amplification primers; (3) as hybridization detection probes to detect the presence of a native protein and/or a related protein in a sample; and (4) as PCR primers to generate particular nucleic acid

10 sequence regions, for example, to generate regions to be probed by hybridization detection probes.

The Sir2 proteins and/or nucleic acid sequences include fragments thereof, (e.g., core domains of Sir2 proteins). Preferably, the nucleic acid contains at least 14, at least 20, at least 27, and at least 45, contiguous nucleic acids of a sequence provided in SEQ

15 ID NO.: 25. Advantages of longer-length nucleic acid include producing longer-length protein fragments having the sequence of a Sir2 protein which can be used, for example, to produce antibodies; increased nucleic acid probe specificity under high stringent hybridization assay conditions; and more specificity for related the SIR2 nucleic acid under lower stringency hybridization assay conditions.

20 Another aspect of the present invention features a purified nucleic acid encoding a Sir2 protein or fragment thereof. Specifically, mSIR2 $\alpha$  (SEQ ID NO: 25), mSIR2 $\gamma$  and/or mSIR2 $\beta$ . Due to the degeneracy of the genetic code, different combinations of nucleotides can code for the same protein. Thus, numerous protein fragments having the same amino acid sequences can be encoded for by difference nucleic acid sequences.

25 In preferred embodiments, the nucleic acid encodes at least 12, at least 18, or at least 54 contiguous amino acids of SEQ ID NO.: 25.

Another aspect of the present invention features a purified nucleic acid having a nucleic acid sequence region of at least 12 contiguous nucleotides substantially complementary to a sequence region in SEQ ID NO.: 25. By "substantially

complementary" is meant that the purified nucleic acid can hybridize to the complementary sequence region in nucleic acid sequences of SEQ ID NO.: 25, under stringent hybridizing conditions. Such nucleic acid sequences are particularly useful as hybridization conditions, only highly complementary nucleic acid sequences hybridize.

- 5 Preferably, such conditions prevent hybridization of nucleic acids having 4 mismatches out of 20 contiguous nucleotides, more preferably 2 mismatches out of 20 contiguous nucleotides, most preferably one mismatch out of 20 contiguous nucleotides. In preferred embodiments, the nucleic acid is substantially complementary to at least 20, at least 27, or at least 45 contiguous nucleotides provided in SEQ ID NO.: 25.

- 10 Another aspect of the present invention features a purified protein having at least about 5-20 contiguous amino acids of an amino acid sequence provided in SEQ ID NOS.: 1, 2, 3, 4, 5, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 26, 27 or 28. By "purified" in reference to a protein is meant that the protein is in a form (e.g., its association with other molecules) distinct from naturally occurring protein. Preferably,
- 15 the protein is provided as substantially purified preparation representing at least 75%, more preferably 85%, most preferably 95% or the total protein in the preparation. In preferred embodiments, the purified protein has at least 5 contiguous amino acids of SEQ ID NO.: 27 or 28.

- Preferred protein fragments include those having functional Sir2 activity (e.g.,
- 20 alter the NAD-dependent acetylation status of histone proteins such as H3 or H4; mono-ADP-ribosylate nuclear proteins such as H2B, H3 or H4), or epitope for antibody recognition. Such protein fragments have various uses such as being used to obtain antibodies to a particular region and being used to form chimeric proteins with fragments of other proteins create a new protein having unique properties, such as a
- 25 longer half-life.

Nucleic acid molecules can be inserted into a construct which can, optionally, replicate and/or integrate into a recombinant host cell, by known methods. The host cell can be a eukaryote or prokaryote and includes, for example, yeast (such as *Pichia pastorius* or *Saccharomyces cerevisiae*) bacteria (such as *Escherichia coli* or *Bacillus*

*subtilis*), animal cells or tissue, insect Sf9 cells (such as baculoviruses infected SF9 cells) or a mammalian cells (somatic or embryonic cells, Chinese hamster ovary cells, HeLa cells, human 293 cells, monkey COS-7 cells and mouse NIH 3T3 cells).

The invention also provides vectors or plasmids containing one or more of each  
5 of the SIR2 genes. Suitable vectors for use in eukaryotic and prokaryotic cells are known in the art and are commercially available or readily prepared by a skilled artisan. Additional vectors can also be found, for example, in Ausubel, F.M., *et al.*, "Current Protocols in Molecular Biology", John Wiley & Sons, Inc. (1999) and Sambrook *et al.*, "Molecular Cloning: A Laboratory Manual," 2nd Ed. (1989), the teachings of which are  
10 incorporated herein by reference in their entirety.

Another aspect of the present invention features a recombinant host cell comprising an isolated nucleic acid molecule of a SIR2 gene operably linked to a regulatory sequence. "Operably linked" is intended to mean that the nucleotide sequence(s) is linked to a regulatory sequence in a manner which allows expression of  
15 the nucleic acid sequence(s). The recombinant host cell is made up of an isolated nucleic acid sequence encoding at least about 5-20 contiguous amino acids provided in SEQ ID NO.: 1, 2, 3, 4, 5, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 26, 27 or 28 and a cell able to express the nucleic acid. Cells containing a functioning Sir2 protein can be used, for example, to screen to agonists or antagonists of Sir2 activity.

20 The nucleic acid molecule can be incorporated or inserted into the host cell by known methods. Examples of suitable methods of transfecting or transforming cells include calcium phosphate precipitation, electroporation, microinjection, infection, lipofection and direct uptake. "Transformation" or "transfection" as used herein refers to the acquisition of new or altered genetic features by incorporation of additional  
25 nucleic acids, e.g., DNA. "Expression" of the genetic information of a host cell is a term of art which refers to the directed transcription of DNA to generate RNA which is translated into a protein. Methods for preparing such recombinant host cells and incorporating nucleic acids are described in more detail in Sambrook *et al.*, "Molecular

Cloning: A Laboratory Manual," 2nd Ed. (1989) and Ausubel, *et al.* "Current Protocols in Molecular Biology," (1999), for example.

The host cell is then maintained under suitable conditions for expression of Sir2. Generally, the cells are maintained in a suitable buffer and/or growth medium or nutrient source for growth of the cells and expression of the gene product(s). The growth media are not critical to the invention, are generally known in the art and include sources of carbon, nitrogen and sulfur. Examples include Luria broth, Superbroth, Dulbecco's Modified Eagles Media (DMEM), RPMI-1640, M199 and Grace's insect media. The growth Media may contain a buffer, the selection of which is not critical to the invention. The pH of the buffered Media can be selected and is generally one tolerated by or optimal for growth for the host cell.

The mSIR2 $\alpha$  gene can be expressed in mammalian or insect cells, any mammalian or insect expression plasmid or viral vector driven by native mSIR2 $\alpha$  promoter can be used, any of other mammalian or insect promoters and enhances, any of viral promoters, or any of artificial inducible promoters can be used. The host cells can be cells of murine, human, or any of other organisms including insects cultured in conventional or specialized *in vitro* culture conditions. The cells transfected with a mSIR2 expression plasmid or infected by the virus containing a mSIR2 $\alpha$  expression vector.

The host cell is maintained under a suitable temperature and atmosphere. Alternatively, the host cell is aerobic and the host cell is maintained under atmospheric conditions or other suitable conditions for growth. The temperature should also be selected so that the host cell tolerates the process and can be, for example, between about 13°-40°C.

The invention also relates to a method of preparing a Sir2 protein using the recombinant host cells described above. The recombinant host cells are biological factories to produce proteins encoded for by the isolated nucleic acid molecule of SIR2.

The chromosomes of higher eukaryotes are compartmentalized into two cytogenetically distinct regions, euchromatin and heterochromatin. Heterochromatin

- was initially described as chromosomal regions that failed to decondense after mitosis (Heitz, E. *Jb. Wiss. Bot.* 69:728(1928)) Subsequent studies have revealed that heterochromatin is formed on highly repetitive DNA, such as centromeres and telomeres, replicates late in the cell cycle and is transcriptionally inactive (Hennig, W.
- 5 *Heterochromatin. Chromosoma* 108:1-9 (1999)). Although many questions about the functional and structural properties of heterochromatin are unresolved, progress in understanding its molecular nature has been made in studies of yeast (Karpen, G., *et al., Trends Genet* 13: 489-496 (1997)); (Grunstein, M. *Cell* 93, 325-328 (1998)) and *Drosophila* (Wakimoto, B. T., *Cell* 93: 321-324 (1998)). In *Drosophila*, genes
- 10 juxtaposed to centromeric heterochromatin through chromosomal rearrangement or transposition become inactivated. The phenomenon, known as position effect variegation (PEV) (Muller, H. J., *J. Genet* 22: 299-335 (1930)), results from the spreading of heterochromatin across the rearrangement breakpoint. Genetic screens have identified large numbers of PEV modifiers some of which have been cloned and
- 15 characterized (Reuter, G., *et al., BioEssays* 14: 605-612 (1992); (Weiler, K. S., *et al., Annu. Rev. Genet.* 29: 577-605 (1995); (Wallrath, L. L., *Curr. Opin. Genet. Dev.* 8: 147-153 (1998)). Heterochromatin protein 1 (HP1) are one of the best-characterized heterochromatin components and known to localize at centromeres and telomeres (James, T. C., *et al., Mol. Cell. Biol.* 6: 3862-3872 (1986); (James, T. C., *et al., Eur. J.*
- 20 *Cell Biol.* 50: 170-180 (1989); (Fanti, L., *et al., Mol. Cell* 2: 527-538 (1998)). HP1-related proteins have been identified from a wide variety of organisms including mammals, and a region termed the chromodomain is highly conserved in HP1 family members (Singh, P. B., *et al., Nucleic Acids Res.* 19: 789-794 (1991)). The chromodomain is also shared by a related set of chromatin factors, the Polycomb group
- 25 (*Pc-G*) (Pirrotta, V., *Cell* 93: 333-336 (1998)). *Pc-G* is required to maintain the repressed state of homeotic genes through many cell divisions in the developmental stages of *Drosophila* (Orlando, V., *et al., Cell* 75: 1187 (1993)).

Transcriptional silencing in budding yeast is associated with genomic domains of structurally altered chromatin akin to heterochromatin in higher cells (Sherman, J. M., *et al.*, *Trends Genet.* 13: 308-313 (1997)).

The principle genes required for silencing *SIR2*, *SIR3*, and *SIR4* encode members of a complex that is targeted to the specific chromosomal domains that are silenced (Ivy, J. M., *et al.*, *Mol. Cell. Biol.* 6: 688-702 (1986); (Gotta, M., *et al.*, *J. Cell Biol.* 134: 1349-1363 (1996); (Rine, J., *et al.*, *Genetics* 116: 9-22 (1987); (Aparicio, O. M., *et al.*, *Cell* 66: 1279-1287 (1991)). For example, silencing at the extra copies of  $\alpha$  and **a** mating type genes at HML $\alpha$  and HM**R****a** is mediated by the binding of these three Sir proteins, plus a fourth, Sir1p, to a set of DNA binding factors including Rap1p, ORC, and Abf1p (Triolo, T., *et al.*, *Nature* 381: 251-253 (1996)).

In contrast, at telomeres Sirs 2p, 3p, and 4p bind to tandem Rap1p molecules arrayed at the telomeric repeat tract (Hardy, C. F. J., *et al.*, *Genes Dev.* 6: 801 (1992); (Moretti, P., *et al.*, *Genes Dev.* 8: 2257 (1994)). Finally, at the rDNA, Sir2p, but not the other Sir proteins, is bound to a specific region in the rDNA spacer in a complex termed RENT which includes another protein, Net1p (Shou, W., *et al.*, *Cell* 97: 233-244 (1999)).

The function of SIR-mediated silencing at HM loci is to prevent the expression of both **a** and  $\alpha$  mating types, which would lead to non-mating, pseudo-diploid strains. The role of the Sir complex at telomeres is less apparent. Interestingly, the telomeric Sir complex, along with the Ku protein, moves to sites of double strand DNA breaks to aid their repair by the non-homologous end joining pathway, suggesting that telomeric Sir complex exists as a poised DNA repairosome (Mills, K. D., *et al.*, *Cell* 97: 609-620 (1999); (Martin, S. G., *et al.*, *Cell* 97: 621-633 (1999)).

Sir2p in the rDNA silences marker genes inserted there (Bryk, M., *et al.*, *Genes Dev.* 11: 255-269 (1997); (Smith, J. S., *et al.*, *Genes Dev.* 11: 241-254 (1997)) and, plays an important role in repressing recombination within the 100-200 tandemly repeated



copies of the rDNA sequences in chromosome XII (Gottlieb, S., *et al.*, *Cell* 56: 771-776 (1989)).

Replicative life span in yeast mother cells (the number of times a mother cell divides to give daughters) is limited, at least in part, by the accumulation of  
 5 extrachromosomal rDNA circles (ERCs) in nuclei of aging mothers (Sinclair, D. A., *et al.*, *Science* 277: 1313-1316 (1997)). These accumulated ERCs result from the excision of circles from the chromosomal rDNA array by homologous recombination (Park, P. U., *et al.*, *Mol. Cell. Biol.* 19: 3848-3856 (1999)), the subsequent replication of ERCs in future cell divisions, and the preferential segregation of ERCs to mother cells.

10 *SIR2*, *SIR3*, and *SIR4* play a key role in determining the life span of yeast mother cells (Kennedy, B. K., *et al.*, *Cell* 89: 381-391 (1997); (Kaeberlein, M., *et al.*, *Genes Dev.* 13: 2570-2580 (1999)). Deleting *SIR3* or *SIR4* shortens the life span by about 25%, and deleting *SIR2* shortens the life span by about 50%. The shortening in the *sir3* and  
 15 *sir4* mutants is due to the simultaneous expression of a and  $\alpha$  mating type information, which somehow causes an increase in recombination in the rDNA leading most likely to an earlier accumulation of a lethal number of ERCs (Kaeberlein, M., *et al.*, *Genes Dev.* 13: 2570-2580 (1999)).

The shortening in *sir2* mutants is due to the absence of Sir2p in the rDNA leading to the elevated rate of recombination at that locus and the rapid generation of  
 20 ERCs. The short life span of *sir2* mutants is suppressed by deletions of *FOB1* (Kaeberlein, M., *et al.*, *Genes Dev.* 13: 2570-2580 (1999)), which encodes a protein that creates a unidirectional barrier to DNA replication in the rDNA (Kobayashi, T., *et al.*, *Genes Cells* 1: 465-474 (1996)). Evidently this barrier is a signal that provokes recombination in the rDNA and the generation of ERCs (Defossez, P. A., *et al.*, *Mol.*  
 25 *Cell* 3: 447-455 (1999)), and the role of Sir2p is to counteract this hyperrecombination. In the absence of Fob1p (and the replication barrier) Sir2p is no longer needed to prevent the formation of ERCs. The addition of a second copy of *SIR2* to cells extends the replicative life span of mothers well beyond that of the wild type, indicating that Sir2p is

the limiting factor for life span (Kaeberlein, M., *et al.*, *Genes Dev.* 13: 2570-2580 (1999)).

Since chromatin at HML and HMR is hypoacetylated (Braunstein, M., *et al.*, *Mol. Cell. Biol.* 16: 4349-4356 (1996); (Braunstein, M., *et al.*, *Genes Dev* 7: 592-604 (1993)), Sir2p was hypothesized to be a histone deacetylase. However, it is also possible that hypoacetylation of chromatin results from some other effect, such as the exclusion of acetylases by the inaccessible structure of these chromosomal regions or the recruitment of other deacetylases.

More recent studies identified SIR2 orthologs in yeast and homologs in many species, including bacteria, which all share a core homology of about 270 amino acids. The four *S. cerevisiae* orthologs *HST1-4* do not play any role in yeast silencing, with the exception that high level expression of *HST1* can partially compensate for the absence of *SIR2* (Brachmann, C. B., *et al.*, *Genes Dev.* 9: 2888-2902 (1995)). The bacterial homolog, *cobB*, can apparently function in cells in a biosynthetic step that transfers phosphoribose from nicotinate mononucleotide to dimethyl benzimidazole (Tsang, A. W., *et al.*, *J. Biol. Chem.* 273: 31788-31794 (1998)). The present invention, documents that yeast Sir2p and its mouse homologue mSir2 $\alpha$  are mono-ADP-ribosyltransferases and NAD hydrolases with specifically acetylated histone substrates. Amino acid changes in mSir2 $\alpha$  that disrupt this enzymatic activity also reduce function *in vivo*. The modification of the acetylation status of N-terminal tails of histones by Sir2 can trigger genomic silencing in yeast and heterochromatin formation more generally in eukaryotic organisms.

As described herein, yeast Sir2p and its mouse Sir2 $\alpha$  homologue possess ADP-ribosyltransferase and NAD hydrolase. These activities are stimulated by N-terminal peptides of histones H3 and H4. In particular, acetylated lysines of histone proteins Lys9Ac and Lys14Ac of H3 and Lys16 of the H4. Transfer to histone H2B is also observed. Studies using enzyme inhibitors and snake venom phosphodiesterase show that Sir2p and mSir2 $\alpha$  are mono-ADP ribosyltransferases.

### Sir2 proteins are novel nuclear mono-ADP-ribosyltransferases with histone substrates

Yeast and mouse Sir2 proteins are shown herein to be mono-ADP-ribosyltransferases by several criteria. First, the proteins transfer  $^{32}\text{P}$ -labeled ADP-ribose from NAD to histones H2B and H3. The endogenous mSir2 $\alpha$  protein immunoprecipitated from NIH3T3 cell extracts shows the same activity. Second, this modification can be removed by snake venom phosphodiesterase, which digests the phosphodiester bond in the ADP-ribose moiety. Third, the reactions are sensitive to mono-ADP-ribosylation inhibitors, but not to poly(ADP-ribose)polymerase (PARP) inhibitors. ySir2p and mSir2 $\alpha$  provide the first examples of nuclear mono-ADP-ribosyltransferases in yeast and mammals.

In the mammalian nucleus, PARPs are likely involved in DNA repair (Menissier-de Murcia, J., *et al.*, *Proc. Natl. Acad. Sci. USA* 94: 7303-7307 (1997); (Wang, Z. Q., *et al.*, *Genes Dev.* 11: 2347-2358 (1997)), replication (Yoshida, S., *et al.*, *Mol. Cell. Biochem.* 138: 39-44 (1994)), transcription (Oliver, F. J., *et al.*, *Embo J.* 18: 4446-4454 (1999)), apoptosis (Germain, M., *et al.*, *J. Biol. Chem.* 274: 28379-28384 (1999)) and telomere maintenance (Di Fagagna, F. D. A., *et al.*, *Nat. Genet.* 23: 76-80 (1999)). On the other hand, it has been reported that alkylating agents induce mono-ADP-ribosylation of histones H1, H2B, and H3 (Kreimeyer, A., *et al.*, *J. Biol. Chem.* 259: 890-896 (1984); (Adamietz, P., *et al.*, *J. Biol. Chem.* 259: 6841-6846 (1984)). Interestingly, many viral, bacterial, and eukaryotic enzymes such as diphtheria, pertusis, and cholera toxins (DT, PT, and CT, respectively) and vertebrate arginine-specific mono-ADP-ribosyltransferases catalyze similar reactions (Domenighini, M., *et al.*, *Mol. Microbiol.* 21: 667-674 (1996)).

These ADP-ribosyltransferases share structural motifs in their catalytic domains and can be classified into two groups: the DT group and the CT group (Domenighini, M., *et al.*, *Mol. Microbiol.* 21: 667-674 (1996); (Moss, J., *et al.*, *Mol. Cell Biochem.* 193: 109-113 (1999)). The DT group includes diphtheria toxin and the family of PARPs, while the CT group includes CT, PT, and other eukaryotic

mono-ADP-ribosyltransferases. ySir2p and mSir2 $\alpha$  share at least one motif of the CT group (see Figure 2C), hydrophobic-Ser-Thr-Ser-hydrophobic (VSTSL, SEQ ID NO: 30, in ySir2p and VSVSC, SEQ ID NO: 31, in mSir2 $\alpha$ ), which forms the NAD-binding cleft. The mutation of the first serine, which is known to be important for positioning

5 NAD, abolishes the mono-ADP-ribosylation activity of the mSir2 $\alpha$  (see Figure 6C).

The mutagenesis of highly conserved residues in the mSir2 $\alpha$  core domain revealed that mutations in 10 of 10 residues affected the mono-ADP-ribosylation activity. In mouse cells, 9 of these 10 mutants were inactive in a transient assay for repression of a reporter, suggesting that the enzymatic activity is critical *in vivo*. The mutant, P285A, 10 could misfold in *E. coli* and thus be inactive. Genetic analysis of the core of yeast Sir2p indicates that the four conserved cysteines at positions 372, 374, 396, and 398 are crucial for silencing *in vivo*. Further, chimeras in which a human Sir2 core domain is substituted for the yeast domain can function in silencing at HM loci, but not at telomeres or rDNA.

#### 15 **Specific acetylation of H3 and H4 tails stimulates Sir2 activities**

A striking feature of the specificity of ADP-ribosylation of H3 was revealed by the use of N-terminal peptides (residues 1-20). Sir2 modified the H3 N-terminal tail peptide when Lys9 and Lys14 were acetylated but not when the peptide was unacetylated.

These lysines are acetylated and important for silencing *in vivo* (Thompson, J. S., *et al.*, 20 *Nature* 369: 245-247 (1994)). In addition, a large fraction of the NAD was hydrolyzed specifically in the presence of the diacetylated peptide and either yeast or mouse Sir2. This latter activity can reflect inefficient transfer of ADP-ribose to the peptide substrate or can present another yet unknown activity of Sir2 proteins in the modification of histones. These data show that the N-terminal tail of H3 is a biologically relevant target 25 of Sir2 proteins *in vivo*.

An H4 peptide (residues 1-20) acetylated on Lys16 stimulated both ADP-ribosyltransferase and NAD hydrolase activities of Sir2, but H4 peptides

acetylated on Lys5, Lys8, or Lys12 did, at best, very weakly. A tetra-acetylated H4 peptide (acetylated Lys5, 8, 12 and 16) was also a poor substrate. Lys16 is a highly acetylated residue in mammalian chromatin. In constitutive heterochromatin, H4 is hypoacetylated at all four lysines, but in facultative heterochromatin Lys5, 8, and 12 are hypoacetylated, while Lys16 is not. Thus, these data illustrate the role of Sir2 in regulating the state of facultative heterochromatin, consistent with the observed intracellular localization of the protein. In yeast mutations of Lys16 to an uncharged residue, glutamine, reduce silencing, and mutation to arginine, even though positive charge is retained, also reduces silencing. Mutations in Lys5, 8, or 12 to arginine, in contrast, exert smaller effects on silencing. These findings are consistent with the idea that the acetylated Lys16 residue of H4 is critical for recognition by Sir2.

ADP-ribosylation of histone N-terminal tails can lead to heterochromatin and gene silencing by interacting with histone residues that are constitutively acetylated, like Lys16 of mammalian H4, thereby inducing ADP-ribosylation by Sir2 proteins in one or more adjacent residues in the N-terminal tail (Figure 7). In active chromatin, the lysines in the N-terminal tails of H3 and H4 are acetylated (depicted by stars at top). Sir2 recognizes H3 and H4 tails containing specifically acetylated lysines (indicated by a large star). Sir2 ADP-ribosylates these N-terminal tails, causing the formation of heterochromatin, perhaps by the recruitment of histone deacetylases.

The ADP-ribosylation in turn facilitates silencing, for example, by restricting access of histone acetylases or by recruiting deacetylases, leading to the hypoacetylated state of other lysine residues in the tail and thus a tighter chromatin structure. ADP-ribosylation and deacetylation are targeted to specific regions of the genome by the selective recruitment of Sir2. In yeast, Sir3p and Sir4p facilitating targeting of Sir2p to telomeres and HM loci, and Net1p and perhaps other components of the RENT complex recruit Sir2p to the rDNA. Consistent with this model, overexpression of *Sir2* induces a more global hypoacetylation of histones H2B, H3 and H4 in yeast.

The model depicted in Figure 7 is consistent with the silencing phenotypes of several yeast mutants. Rpd3p is a histone deacetylase. Rpd3 mutants show

hyperacetylation of histones and generally an increase in transcription of genes assayed . An exception to this rule, however, occurs in the case of silenced chromatin at yeast telomeres and rDNA, where silencing actually increases in the mutant. The increase in silencing can occur as a result of hyperacetylation of the lysines in the N-terminal tail  
 5 that are recognized by Sir2. *NAT1/ARD1* encode subunits of an N-terminal acetyltransferase. Mutations in either gene abolish silencing in yeast. It is possible that the *nat1* or *ard1* mutants have underacetylation of histone residues that potentiate Sir2 activity. Additionally, or alternatively, the effects of these mutants can be indirect.

### Mammalian Sir2 proteins

10 *SIR2* homologs have been found in a variety of organisms. Human *SIR2* homologs that all correspond to the same gene, mSIR2 $\beta$ , which is most related to yeast HST2 have been reported (Afshar, *et al.*, *Gene* 234:161-168(1999); Frye, *Biochem. Biophys. Res. Commun.* 260:273-279(1999); Sherman, *et al.*, *Mol. Biol. Cell* 10:3045-3059 (1999)). In contrast, the human SIR2 gene appears to correspond to a fragment of  
 15 a homolog of mSIR2 $\alpha$ . Of all mammalian *SIR2* homologs analyzed to date, mSIR2 $\alpha$  shows the greatest homology to ySir2p in the evolutionarily conserved core domain. Nonetheless, there are some significant differences between ySir2p and mSir2 $\alpha$ . mSir2 $\alpha$  has a diverged N-terminal portion and a longer C-terminal tail and is almost twice the size of ySir2p. It is possible that these mSir2 $\alpha$ -specific regions may be  
 20 involved in species-specific or cell type-specific protein-protein interactions.

In yeast, the Sir complex moves to sites of DNA double strand breaks. Since ADP-ribosylation has been associated with sites of DNA repair in mammalian cells, it is likely that mammalian Sir2 proteins are recruited to DNA damage. Mono-ADP-ribosylation of histones near sites of DNA damage in yeast and mammals likely  
 25 have an important biological role.

### Link between energy metabolism, heterochromatin and aging

The substrate for Sir2 modification of chromatin, NAD, may be an indicator of the energy status of cells. Cells grown under energy limitation may have a high NAD/NADH ratio, while cells growing under energy surplus would have a low ratio.

- 5 Since NAD but not NADH is expected to function as a substrate in the mono-ADP-ribosylation reaction, Sir2 activity can be highest under conditions of energy limitation. Thus, the rate of energy metabolism may control gene expression of certain chromosomal domains by regulating the activity of Sir2.

- Recent studies indicate that Sir2p is a limiting component in the determination of the life span of yeast mother cells for example, overexpression of Sir2p extends the life span beyond wild type. Caloric or dietary restriction is associated with an extension of life span in rodents, *C. elegans*, and even yeast. Caloric restriction may exert of its effect, in part, by creating a condition of energy limitation, which would activate Sir2 and promote ADP-ribosylation of chromatin and gene silencing. ADP-ribosylation is a biochemical event which has been suggested to play a role in aging. Alterations in the maintenance of Sir2-silenced chromatin could result in normal mammalian aging and by slowing caloric restriction. The fact that Sir2 proteins are simple enzymes suggests a strategy for intervening in non genetic ways to influence life span. Small molecules that are agonists for this enzyme can extend life span in yeast cells and in mammals.

- 20 The present invention shows that yeast Sir2p and its mouse homolog mSir2 $\alpha$  are ADP-ribosyltransferases and NAD hydrolases with specific histone substrates, the N-terminal tails of H3 and H4. Both activities are stimulated by acetylation in the histone tails, in particular Lys9Ac and Lys14Ac for H3, and Lys16Ac for H4. Acetylation of these lysine residues is important for silencing of chromatin *in vivo*. Mutations in the conserved sequences of mSir2 $\alpha$  reduce ADP-ribosyltransferase activity *in vitro* and chromatin of the silencing activity in mouse NIH3T3 cells. The mSir2 $\alpha$  protein is localized in the nuclei of mouse cells shown that acetylation –terminal tails of H3 and H4 trigger ADP-ribosyltransferase and NAD hydrolase activities of Sir2 proteins. The

ADP-ribosylation and acetylation of histones proteins can be important in the formation of heterchromatin in eukaryotic cells.

The present invention shows that Sir2 proteins are novel NAD-dependent deacetylases specific for the N-terminal tail of histone H3. Although NAD and NADH  
5 are frequent enzyme cofactors in oxidation/reduction reactions, this is the first time NAD has been shown to drive a distinct enzymatic reaction in a substrate, such as a peptide of the amino-terminal tail of histone H3 di-acetylated at lysines 9 and 14.

Since histones in silenced chromatin are hypoacetylated, and because overexpressed Sir2p promotes global deacetylation of histones in yeast (Braunstein, M.,  
10 *et al.*, *Genes Dev* 7: 592-604 (1993); (Braunstein, M., *et al.*, *Mol. Cell. Biol.* 16: 4349-4356 (1996)), it was originally proposed that Sir2p might be a histone deacetylase. However, such an activity could not be demonstrated until now, presumably because of its absolute requirement for NAD. The present invention is consistent with studies that show mutating lysines in the tails of histones H3 and H4 can reduce silencing *in vivo*  
15 (Thompson, J. S., *et al.*, *Nature* 369: 245-247 (1994); (Braunstein, M., *et al.*, *Mol. Cell. Biol.* 16: 4349-4356 (1996)) and suggest that this NAD-dependent deacetylation of histones *per se* is sufficient to establish silencing *in vivo*. These data show that *SIR2* mutations reduce or eliminate deacetylase activity show corresponding defects in silencing, suppression of recombination, and life span *in vivo*. The nature of the  
20 requirement of NAD for deacetylation is not yet clear, but at the concentrations of about one hundred  $\mu$ M, NAD may serve as an allosteric effector to activate the histone deacetylase activity of Sir2. The failure of NADH, NADP, or NADPH to function as such reveals a remarkable specificity of Sir2p for the oxidized form of NAD. Sir2 proteins catalyze the putative transfer of ADP-ribose from NAD to the proteins  
25 such as intact histone H3 or the amino-terminal peptide and labeled NAD, to allow the detection of low levels of  $^{32}$ P transfer. However, in reactions allowing a direct comparison between ADP-ribosylation and deacetylation (using the H3 peptide at 1 mM NAD), the reaction products displayed at least 27% conversion of the peptide to deacetylated species, and no detectable conversion to any ADP-ribosylated species.



Thus, ADP ribosyltransferase reaction proceeds to a much lesser degree than deacetylation, at least with this H3 peptide substrate.

Coumermycin A1 is an inhibitor of mono-ADP-ribosyltransferases (Banasik, M., *et al.*, *Mol. Cell. Biochem.* 138: 185-187 (1994)) and was shown to inhibit the putative ADP-ribose transferase activity as well as the accompanying NAD hydrolysis. However, coumermycin A1 did *not* inhibit the Sir2p deacetylase activity at all. These findings indicate that the putative ADP-ribosyltransferase activity is separable and may play a distinct role *in vivo*. The ADP-ribosylation of histones is known to occur when cells are treated with DNA damaging agents (Adamietz, P., *et al.*, *J. Biol. Chem.* 259:6841-6846 (1984); (Kreimeyer, A., *et al.*, *J. Biol. Chem.* 259: 890-896 (1984); (Pero, R. W., *et al.*, *Mutat. Res.* 142: 69-73 (1985)). Moreover, antibodies against mono-ADP-ribose react with mammalian nuclei only if when cells are treated with DNA-damaging agents. Thus ADP-ribosylation of histones and NAD hydrolysis may play a role in opening up chromatin to allow DNA repair. In this regard, the Sir proteins are known to move to sites of DNA breaks to aid their repair by non-homologous end joining (Mills, K. D., *et al.*, *Cell* 97: 609-620 (1999); (Martin, S. G., *et al.*, *Cell* 97: 621-633 (1999)).

There are four *SIR2*-related genes in yeast, *HST1-4* (Brachmann, C. B., *et al.*, *Genes Dev.* 9: 2888-2902 (1995)), but no single *hst* mutant affects silencing, although effects are observed in the *hst3/hst4* double mutant. It is expected that these gene products catalyze histone deacetylation. In mammals there are at least five homologs (Frye, R. A., *Biochem. Biophys. Res. Commun.* 260: 273-279 (1999); (Brachmann, C. B., *et al.*, *Genes Dev.* 9: 2888-2902 (1995)), of which mSir2 $\alpha$  is the most closely related to Sir2p. mSir2 $\alpha$  is identical to yeast Sir2p in the deacetylation reaction, indicating that this activity is highly conserved in nature.

mSir2 $\alpha$  is not at the rDNA, centromeres, or telomeres, but broadly distributed in nuclei (not shown) suggesting that Sir2 proteins may regulate silencing in widely distributed blocks of the mammalian genome. A distinction between Sir2-silenced

heterochromatin and constitutive heterochromatin is illustrated by the disruption of centromeric heterochromatin in *S. pombe* by the general deacetylase inhibitor, trichostatin A (TSA) (Ekwall, K., *et al.*, *Cell* 91: 1021-1032 (1997)), which does not affect the deacetylase activity of Sir2p. The involvement of a distantly related *SIR2* family member, *hst4+*, in this centromeric silencing (Freeman-Cook, L. L., *et al.*, *Molecular Biology of the Cell* 10: 3171-3186 (1999)) provides a hint that the more distant *SIR2* family members may target constitutive heterochromatin, while closely related *SIR2* genes target regulated blocks of silencing.

In addition to a role in silencing, Sir2p also extends life span of mother cells by repressing recombination in the rDNA (Gottlieb, S., *et al.*, *Cell* 56: 771-776 (1989)), and reducing the production of toxic rDNA circles (Sinclair, D. A., *et al.*, *Science* 277: 1313-1316 (1997); (Sinclair, D. A., *et al.*, *Cell* 91: 1-20 (1997)). The fact that NAD but not NADH, NADP, or NADPH can activate deacetylation of histones by Sir2p may link chromatin silencing and ageing to the metabolic state of cells, i.e., Sir2 proteins can sense the energy or oxidation state of cells. Most of the NAD in eukaryotic cells is present in the nucleus (Rechsteiner, M., *et al.*, *Nature* 259: 695-696 (1976)), where it turns over rapidly (Dai, Y., *et al.*, *Mut. Res.* 191: 29-35 (1987)). Thus, a pool of NAD can regulate chromatin structure by controlling the histone deacetylase activity of Sir2 proteins.

It is interesting to note that caloric restriction slows ageing in a variety of organisms, including yeast (Muller, I., *et al.*, *Mech. Ageing Dev.* 12: 47-52 (1980)), *C. elegans* (Lakowski, B., *et al.*, *Proc. Natl. Acad. Sci. USA* 95: 13091-13096 (1998)), rodents (Weindruch, R. H., *et al.*, *J. Nutr.* 116: 641-654 (1986)), and probably primates (Roth, G. S., *J. Am. Geriatr. Soc.* 47: 896-903 (1999)). It is possible that the altered metabolic rate in calorically restricted cells exert at least part of its effect by increasing the availability of NAD in the nucleus, which, in turn, up-regulates Sir2 proteins and chromatin silencing. The hunkering down of the genome in the face of carbon limitation is sensible because it conserves energy and could explain the extended life span under

this regimen. In normal ageing, NAD levels decline (Chapman, M. L., *et al.*, *Mech. Of Aging and Dev.* 21: 157-167 (1983)), perhaps causing a decrease in Sir2 activity and a deleterious loss of genomic silencing.

- It is clear that nuclei obtained from adult animals may be reprogrammed in
- 5 oocytes to undergo embryonic development and produce viable progeny (Wilmot, I., *et al.*, *nature* 385: 810-813 (1997); (Wakayama, T., *et al.*, *Nature* 394: 369-374 (1998)). This cloning implies that there can be no irreversible changes in nuclei of ageing animals, such as DNA deletions or DNA circles. Were changes in chromatin structure the basis of ageing, then such a reversibility in oocytes would be theoretically possible.
- 10 It is possible that reprogramming of adult nuclei, a slow and inefficient process, can be facilitated by the addition of chromatin modification factors, such as Sir2 to oocytes. Moreover, it possible that increasing the activity of Sir2 in metazoans extends their life span, as it does in yeast (Kaeberlein, M., *et al.*, *Genes Dev.* 13: 2570-2580 (1999)). The most ready way to achieve this goal is to provide additional copies of *SIR2* genes in
- 15 transgenic animals. A more long term strategy with possible implications to humans is to intervene with small molecules that increase the deacetylation activity of Sir2. The latter approach offers the advantage of late intervention to avoid any unwanted side effects on development or fertility and could, in principle, afford the benefits of caloric restriction without the practical difficulties.
- 20 While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims. The teachings of all references cited herein are hereby incorporated by reference in their entirety.

## EXEMPLIFICATION

## MATERIALS AND METHODS

**Data base and sequence analysis**

TBLASTN searches were performed on the NCBI mouse EST sequence databases, using the amino acid sequence of ySir2p. All mouse EST sequences homologous to ySir2p were classified into three groups termed  $\alpha$ ,  $\beta$ , and  $\gamma$ , based on the homology results from the searches. Three representative EST cDNA clones were purchased for three mouse homolog groups from Genome Systems Inc (St. Louis, MO): AA199012 for  $\alpha$ , AA105536 for  $\beta$ , and AA260334 for  $\gamma$ . The cDNA clones were partially or completely sequenced. All deduced amino acid sequences were aligned with the Clustal X program. To cover each core domain completely, amino acid sequences of AA137380 and AA212772 for  $\beta$  and  $\gamma$  respectively, were also used. A phylogenetic tree of the core domains of the yeast and mouse *Sir2* families was generated with the Clustal X and NJPLOT program by using the following amino acid sequences: position 228-499 for ySir2p, 174-440 for yHst1p, 1-251 for yHst2p, 26-315 for yHst3p, 65-343 for yHst4, 215-460 for mSir2 $\alpha$ , TLGL to LINKEK (SEQ ID NO: 32) for mSir2 $\beta$ , and FGGG to LINRDL (SEQ ID NO: 33) for mSIR2 $\gamma$ .

**Northern blotting**

The multiple tissue Northern blot (heart, brain, spleen, lung, liver, skeletal, muscle, kidney, testis) was purchased from Clontech (Palo Alto, CA). Prehybridization and hybridization were performed at 65°C in 5X SSPE, 5X Denhardt's solution, 1% SDS and 0.1mg/ml poly(A). The cDNA fragments of AA199012, AA105536, and AA260334 were used as probes for mSIR2 $\alpha$ ,  $\beta$ , and  $\gamma$ , respectively. The human  $\beta$  actin fragment from the manufacturer was used as a control probe. Between each blotting, the probe was stripped off by boiling the membrane in 0.5% SDS for 1min.

### Molecular cloning of mSIR2 $\alpha$

The mouse 15-day embryo 5'-STRETCH PLUS cDNA library (Clontech) was screened with the cDNA fragment of AA199012 as a probe. Five positive clones were obtained from approximately one million independent plaques. One of the five clones  
 5 contained a 3.9kb cDNA fragment. The nucleotide sequence of this fragment was determined with an Applied Biosystems 374 automated sequencer. Although more 5' sequences of mSIR2 $\alpha$  cDNA were obtained by 5' RACE with the mouse liver Marathon-Ready cDNA (Clontech), no stop codon could be found in the upstream of the first start codon (data not shown). This finding was confirmed on the genomic sequence  
 10 of the mSIR2 $\alpha$  gene (data not shown). Thus, the cDNA clone encodes the full-length mSir2 $\alpha$  protein, as shown in Figure 2B. The deduced amino acid sequence of mSir2 $\alpha$  was aligned in the Clustal X and SeqVu 1.1 programs with other *Sir2* family members.

### Antibody production to mSir2 $\alpha$

The 5' Sall-PvuI fragment of the mSIR2 $\alpha$  cDNA was engineered to be cloned into  
 15 BamHI site of pET16b vector (Novagen, Madison, WI). The BL21(DE3)pLysS bacterial strain that also has an extra copy of arginine tRNA gene was transformed with the resultant plasmid. A transformed bacterial clone was induced in 1mM IPTG at 37°C for 7hrs to produce 10XHis-tagged N-terminal fragment of the mSir2 $\alpha$  protein. The mSir2 $\alpha$  N-terminal protein was purified with Ni-NTA agarose (QIAGEN, Valencia, CA)  
 20 under denaturing condition. Rabbit polyclonal antisera against this purified protein was produced at Covance Research Products (Denver, PA). Affinity purification of the antibody was performed with HiTrap NHS-activated column (Amersham Pharmacia Biotech, Piscataway, NJ) conjugated with the dialyzed mSir2 $\alpha$  N-terminal protein.

### Western blotting and immunoprecipitation

25 Mouse NIH3T3 cells were lysed directly in Laemmli's sample buffer for Western blotting or in extraction buffer (20mM Tris-HCl [pH7.6], 150mM NaCl, 0.5% IGEPAL

CA-630 (SIGMA, St. Louis, MO), 1mM EDTA, 0.5mM PMSF, 10µg/ml leupeptin, 10µg/ml pepstatin A, 10µl/ml aprotinin) for immunoprecipitation. The *in vitro* translated mSir2α protein was produced with the XbaI-linearized pBluescript containing the mSir2α cDNA at SalI site and the STP3 T7 *in vitro* transcription/translation kit (Novagen). For Western blotting, 17µg of the extract or 2µl of the *in vitro* translation mixture was run on a 4-15% gradient SDS-PAGE gel and transferred onto an Immobilon-P PVDF membrane (Millipore, Bedford, MA). After preblocking strips of the membrane in TBS containing 0.1% Tween20 and 5% nonfat skim milk, the first antibody reaction was performed in the 1:2000 dilution of the affinity-purified antibody against mSir2α or control rabbit IgG. The mSir2α band was visualized with the secondary anti-rabbit IgG antibody conjugated with horse radish peroxidase and the ECL detection kit (Amersham Pharmacia Biotech). For immunoprecipitation, approximately 300µg of the extract was incubated at 4°C with 1µg of the affinity-purified mSir2α antibody for 1hr and then with Protein A-Sepharose beads (SIGMA) for another 1hr. The protein complex on beads was washed with extraction buffer three times and extracted in sample buffer. The protein was electrophoresed and blotted as described above.

### Immunofluorescence

NIH3T3 cells or mouse embryonic fibroblasts were plated on 10-well multitest slideglasses (ICN Biomedicals, Aurora, OH). The cells were briefly washed with PBS, fixed in PBS containing 3.2% paraformaldehyde for 10 min, and then treated with PBS containing 0.5% IGEPAL-CA630 for 20 min. Preblocking reaction was performed with PBG buffer (PBS containing 0.2% cold water fish gelatin and 0.5% bovine serum albumin (SIGMA)) at 37°C for 20min. After washing cells in PBS-T (PBS containing 0.2% Tween20) briefly, the first antibody reaction was performed at 37°C for 1hr in PBG containing the affinity-purified mSir2α antibody diluted to 1:500 and/or one of the anti-human nuclear antibodies (ANA-N for nucleolus or ANA-C for centromeres,

SIGMA) diluted to 1:5 or 1:10 or control rabbit IgG. The cells were washed in PBS-T for 5min twice. The secondary antibody reaction was performed at 37°C for 1hr with the anti-rabbit IgG antibody conjugated with FITC (Jackson ImmunoResearch Laboratories, PA) and/or the anti-human IgG antibody conjugated with Texas-Red (Vector

- 5 Laboratories, CA). After washing in PBS-T for 5min, the cells were counterstained in 200ng/ml of DAPI for 1min. They were washed in TBS-T for 5min again and in dH<sub>2</sub>O briefly, and then embedded under coverslips with Vectorshield (Vector Laboratories).

- For the immuno-FISH of mSir2 $\alpha$  and telomeres, the first antibody reaction for mSir2 $\alpha$  was performed as described above. The cells with the first immune complex
- 10 was then fixed with 3.2% paraformaldehyde for 10 min, permeabilized again in PBS containing 0.5% IGEPAL-CA630 for 20 min, treated in 0.1M NaOH for 2min to denature DNA, and immediately washed in ice-cold PBS. The synthesis of a telomeric probe, hybridization of the probe and visualization of the hybridized spots were done as described previously. The secondary antibody reaction to detect mSir2 $\alpha$  was also done
  - 15 during the last visualization step.

All immunofluorescent digital images were obtained with the Nikon Eclipse TE300 fluorescent microscope equipped with a CCD digital camera (Hamamatsu Photonics, Japan) and the Metamorph imaging system software (Universal Imaging Corp., PA).

## 20 **Production of recombinant proteins**

- The yeast *SIR2* gene or the mSIR2a full-length cDNA was cloned into pET28a vector (Novagen). BL21(DE3) and BL21(DE3)pLysS with an extra copy of arginine tRNA gene was transformed with the ySIR2 and mSIR2 $\alpha$  plasmids, respectively. Each transformed bacterial clone was induced in 1mM IPTG at 37°C for 1hr. The induced
- 25 6XHis-tagged proteins were purified with Ni-NTA agarose under native condition (see Figure 4A). The N-terminal fragment of mSir2 $\alpha$  was prepared in the same way. The

control elute was prepared from a bacterial clone carrying pET28a vector only. The recombinant proteins were aliquoted and kept at  $-70^{\circ}\text{C}$ .

Site-directed mutagenesis of mSir2 $\alpha$  core domain was performed with the GeneEditor system (Promega, Madison, WI) according to the procedure provided by the

- 5 manufacturer. The mutant recombinant proteins were prepared in the same way as described above.

### ADP-ribosylation assay

The typical reaction was performed in 50 or 100 $\mu\text{l}$  of the buffer containing 50mM Tris-HCl [pH8.0 or 9.0], 4mM  $\text{MgCl}_2$ , 0.2mM DTT, 1 $\mu\text{M}$  cold  $\beta\text{-NAD}^+$  (SIGMA) and  
 10 8 $\mu\text{Ci}$  nicotinamide adenine dinucleotide 5'-[ $\alpha\text{-}^{32}\text{P}$ ]triphosphate (NAD) as a donor of ADP-ribose ( $\sim 1000\text{Ci/mmol}$ , Amersham Pharmacia Biotech). To check the sensitivity to poly- or mono-ADP-ribosylation inhibitors, 3-aminobenzamide (ICN Pharmaceuticals, CA), benzamide, novobiocin and coumermycin A1 (SIGMA) were added to the reactions prior to adding Sir2 proteins, respectively. The wild type or  
 15 mutant recombinant proteins (0.5-1 $\mu\text{g}$  for ySir2p and 10 $\mu\text{g}$  of mSir2 $\alpha$ ) were added with 4 $\mu\text{g}$  of each histone purchased from Roche Molecular Biochemicals (Indianapolis, IN). For the peptide experiments, 5-10 $\mu\text{g}$  of unacetylated or acetylated N-terminal tail peptides (amino acids 1-20) of H3 and H4 purchased from Upstate Biotechnology (Lake Placid, NY) were used as substrates of the yeast and mouse Sir2 recombinant proteins.

- 20 To detect the activity of the endogenous mSir2 $\alpha$ , which was usually immunoprecipitated from 300-400 $\mu\text{g}$  of the NIH3T3 whole cell extract, the reaction buffer and substrates were added directly to the immune complex on Protein A-Sepharose beads. The reaction mixture was incubated at room temperature for 1hr. For the reaction of snake venom phosphodiesterase (Roche Molecular Biochemicals),  
 25 1-2 $\mu\text{l}$  of the enzyme or the same volume of  $\text{dH}_2\text{O}$  was added to the reaction mixture, respectively, after finishing ADP-ribosylation reaction. Each mixture was incubated at  $37^{\circ}\text{C}$  for 1hr and the reaction was terminated as described below.



To analyze histone modification, 25 $\mu$ l of 100% trichloroacetic acid (TCA) solution was mixed to the reaction mixture and left on ice for 15min. The protein precipitates were centrifuged at 4°C and washed with 5 or 20 % TCA solution twice. The pellets were dissolved in 15 $\mu$ l of Leammli's sample buffer and boiled for 1.5min.

- 5 The proteins were electrophoresed in a 10-20% gradient SDS-PAGE gel. The gel was stained with Coomassie Brilliant Blue R-250 (GibcoBRL Life Technologies, Frederick, MD) to check equal loading of histones, dried, and exposed to Kodak X-OMAT film.

- 10 To analyze modified peptides, 10 $\mu$ l of each reaction mixture was spotted on a cellulose TLC plate (EM Science, Gibbstown, NJ). The chromatography was performed for 9-10hrs in a TLC chamber containing the 65:5:3:2:29 mixture of isobutyric acid, pyridine, acetic acid, butanol and water . The plate was dried and exposed to Kodak X-OMAT film. The peptide spots were visualized by ninhydrin staining.

### **Transient transfection assay**

- Effector plasmids of mSIR2 $\alpha$  were constructed using DNA fragments corresponding to amino acids 220-500 of the wild type and mutant mSIR2 $\alpha$  amplified by PCR with PfuTurbo DNA polymerase (Stratagene, La Jolla, CA) with primers that create EcoRI sites at both ends of each fragment. Fragments were cloned into the EcoRI site of pM mammalian expression vector (Clontech) to produce the N-terminal fusion protein to the GAL4 DNA binding domain. The luciferase plasmid, pUAS<sub>4</sub>tkluc, which has four GAL4 binding sites in the upstream of the luciferase gene, was employed as a reporter gene. Plasmids preparations were made using the QIAfilter plasmid midi kit (QIAGEN).

- NIH3T3 cells (10<sup>6</sup> cells) were plated a day before transfection. Cells were transfected using SuperFect transfection reagent (QIAGEN) with 4 $\mu$ g of the GAL4DBD-core domain effector plasmid, 1 $\mu$ g of the luciferase reporter gene and 1.5 $\mu$ g of SV40 promoter-driven  $\beta$ -galactosidase gene to normalize the transfection efficiency. The extracts of the transfectants were prepared 48hrs after transfection. The luciferase

assay was performed using the luciferase assay system kit (Promega) and the Optocomp I luminometer (MGM Instruments, CT), according to the procedures provided by the manufacturers.

### Production of recombinant proteins

5        The yeast *SIR2* gene or the *mSIR2 $\alpha$*  full-length cDNA were cloned into pET28a vector (Novagen, WI). BL21(DE3) and BL21(DE3)pLysS with an extra copy of arginine tRNA gene was transformed with the *SIR2* and *mSIR2 $\alpha$*  plasmids, respectively. Each transformed bacterial clone was induced in 1mM IPTG at 37°C for 1hr. The induced 6XHis-tagged proteins were purified with Ni-NTA agarose under native  
10        conditions. The control elute was prepared from a bacterial clone carrying pET28a vector only. The recombinant proteins were aliquoted and kept at -70°C.

### Deacetylation and ADP-ribosylation assays

      The typical reaction of Sir2 deacetylase activity was performed in 50  $\mu$ l of buffer containing 50mM Tris-HCl [pH 9.0], 4mM MgCl<sub>2</sub>, 0.2mM DTT, variable concentration  
15        of cold nicotinamide adenine dinucleotide (NAD) or NAD derivatives (SIGMA, MO), 5-10 $\mu$ g of the purified recombinant Sir2 proteins, and 10 $\mu$ g of the histone H3 N-terminal tail peptide (amino acid 1-20) di-acetylated at positions 9 and 14 (Upstate Biotechnology, NY). This starting peptide material contains a contaminant with 100 Da smaller molecular weight, which also showed exactly the same patterns of deacetylation  
20        (data not shown). To detect the ADP-ribosylation activity, 8 $\mu$ Ci of NAD 5'-[ $\alpha$ -<sup>32</sup>P]triphosphate (~1000Ci/mmol, Amersham Pharmacia Biotech, NJ) was added to the same reaction containing 1 $\mu$ M cold NAD. Histone H3 protein (4 $\mu$ g) (Roche Molecular Biochemicals, IN) were used for this assay. All reaction mixtures were incubated at room temperature for 1hr. Trichostatin A and coumermycin A1 (SIGMA) were  
25        prepared in dimethylsulfoxide (DMSO, SIGMA), and 5 $\mu$ l of solvent or inhibitor was added to the reactions prior to adding Sir2 proteins.

### Analysis of deacetylated or ADP-ribosylated products

After the incubation, the products were precipitated at -20°C overnight by adding 50µl of distilled water and 25µl of 100% trichloroacetic acid (TCA) solution. For high pressure liquid chromatography (HPLC), the precipitates were reconstituted in 5%

- 5 CH<sub>3</sub>CN and 0.1% trifluoroacetic acid (TFA) and run in the gradient concentration between 0.05% TFA and 0.043% TFA plus 80% CH<sub>3</sub>CN on Hewlett Packard Model 1100 HPLC system with 214TP52 column (VYDAC, CA). The chromatograms at the absorbance of 210nm were digitally recorded and analyzed by Hewlett Packard ChemStation system (version A.06.03[509]). Fractions of samples were collected every
- 10 1min by Gilson Fraction Collector Model 203. Peptide sequencing was done by the Applied Biosystems Procise 494 HT protein sequencing system. PTH amino acid chromatograms were recorded and analyzed by the ABI Model 610A2.1 data integration/analysis system.

- Electron-spray mass spectroscopy (also referred to herein as mass spectroscopy)
- 15 was done on the PE Sciex Model API365 system. Matrix assisted laser desorption/ionization (MALDI) mass spectroscopy can also be used. The electron-spray mass spectroscopy data were analyzed by the BioMultiView program (version 1.3.1). To detect the ADP-ribosylated intact H3 protein, the pellets were dissolved in 15µl of Leammli sample buffer, boiled for 1.5min and electrophoresed in a 10-20% gradient
- 20 SDS-PAGE gel. The gel was stained with Coomassie Brilliant Blue to check equal loading of histones, dried, and exposed to Kodak X-OMAT film. To analyze the ADP-ribosylated H3 peptides, 10µl of each reaction mixture was spotted on a cellulose TLC plate (EM Science, NJ). The chromatography was performed for 9-10hrs in a TLC chamber containing the 65:5:3:2:29 mixture of isobutyric acid, pyridine, acetic acid,
- 25 butanol and water (Scheidtmann, K. H., *et al.*, *J. Virol.* 44: 116-133 (1982)). The plate was dried and exposed to Kodak X-OMAT film. The peptide spots were checked by ninhydrin staining.

### Molecular cloning of mSIR2 $\alpha$

The mouse 15-day embryo 5-STRETCH PLUS cDNA library (Clontech, CA) was screened with the EST cDNA fragment of AA199012 as a probe. Five positive clones were obtained from approximately one million independent plaques. One of the five clones contained a 3.9kb cDNA fragment. The nucleotide sequence of this fragment was determined with an Applied Biosystems 374 automated sequencer. The isolated cDNA clone encodes the full-length mSir2 $\alpha$  protein since the *in vitro*-translated protein from this cDNA clone showed an indistinguishable size (110-120kD) to the protein in the mouse NIH3T3 extract recognized by a specific polyclonal antibody against a N-terminal portion of mSir2 $\alpha$  (data not shown).

The amino acid sequences of mSir2 $\alpha$  and other *Sir2* family members were aligned in the Clustal X program and a phylogenetic tree was generated by using the NJPLOT program.

### Strains, plasmids, and antibodies

All strains used were derivatives of W303a *sir2 $\Delta$* : W303R *sir2 $\Delta$* (*MATa*, *ade2-1*, *leu2-3,112*, *trp1-1*, *ura3-52*, *his3-11*, *sir2::TRP1*, *rDNA-ADE2*), W303RT *sir2 $\Delta$* (*MATa*, *ade2-1*, *leu2-3,112*, *trp1-1*, *ura3-52*, *his3-11*, *rad5-535*, *sir2::TRP1*, *rDNA-ADE2*, *URA3-TEL*??), and W303R *sir2 $\Delta$* /*rdp3 $\Delta$* (*MATa*, *ade2-1*, *leu2-3,112*, *trp1-1*, *ura3-52*, *his3-11*, *rdp3::URA3*, *sir2::TRP1*, *rDNA-ADE2*). Two integrating plasmids that contain *SIR2* driven by its native promoter were used: pRS305-SIR2 and pRS305-SIR2\*, the latter expressing Sir2p at low levels. *SIR2* and mutant *sir2* strains were generated by cutting the plasmid within the *LEU2* gene and integrated using standard yeast transformation protocols. Unless otherwise noted derivative strains were generated using pRS305-SIR2. Rabbit antibodies to Sir2p were generated by using full-length rSir2p isolated under denaturing conditions.

### Generation of core domain mutants

Site directed mutations were generated in the plasmid pRS305-SIR2\* using the Gene Editor system (Promega, Madison, WI) according to the procedure provided by the manufacturer. Sequences were verified by Sanger sequencing methods. The mutants  
5 were then subcloned into pRS305-SIR2 and pET28a.

### ADP-ribosylation and deacetylation assays

BL21 with *SIR2* or the *sir2* mutants subcloned into pET28a (Novagen) were induced with 1mM IPTG for 1 hr. The recombinant proteins were purified by Nickel-NTA column under native condition. Ribosylation and deacetylation assays were  
10 performed using 1 µg of recombinant protein for the ribosylation assay and 5µg for the deacetylation assay.

### Silencing, life span and rDNA recombination assays

To evaluate silencing at the telomeres and rDNA, 10-fold dilutions of the derivatives of either W303RT or W303R *Ærpd3* were spotted on media containing 5-  
15 FOA or media lacking adenine, respectively. To assay for HM silencing, W303R derivatives generated with pRS305-SIR2\* were patched onto YPD with the tester strain CKy20(*MATa*, *arg1*, *tsm11*) and after overnight growth were replica plated to minimal media with no supplemented amino acids. Life span and rDNA recombination rates were measured as in Kaerberlein, *et. al.*, (Kaerberlein, M., *et al.*, *Genes Dev.* 13: 2570-  
20 2580 (1999)).

## RESULTS

### Characterization of the closest mouse *SIR2* homolog, mSIR2α

At least three different sequences, mSir2α, β, and γ, related to yeast Sir2p (ySir2p) have been identified in the mouse EST database. mSir2α was identified as having the

greatest homology to ySir2p and the related yeast protein, Hst1p (Figure 1A). mSir2 $\beta$  and  $\gamma$  have significant homology to Hst2p, another member of yeast *Sir2* family.

The tissue distribution of mSIR2 $\alpha$ ,  $\beta$ , and  $\gamma$  mRNA expression was examined (Figure 1B). A major transcript encoding mSIR2 $\alpha$  (~4.0kb) is present in all tissues  
5 examined. Minor transcripts of various size are also detected in each tissue. mSIR2 $\beta$  has only one transcript (~1.9kb). mSIR2 $\alpha$  and  $\beta$  appear to be expressed ubiquitously, showing a high level of expression in liver. The mSIR2 $\gamma$  transcript (~1.6kb) is also predominantly expressed in liver.

A 3.9kb cDNA clone of mSIR2 $\alpha$  was identified by screening a mouse embryonic  
10 cDNA library with the EST clone AA199012 as a probe. This cDNA clone encodes a predicted protein with 737 amino acids with a molecular weight of 80.3kD. The deduced amino acid sequence of mSIR2 $\alpha$  is shown in Figure 2A. A predicted nuclear localization signal KRKKRK (SEQ ID NO: 29) is present.

A rabbit polyclonal antibody raised against the N-terminal 131 amino acids of the  
15 mSir2 $\alpha$  protein recognized a 120kD protein in the cell extracts of murine NIH3T3 cells (Figure 2B) and embryonic fibroblasts (data not shown). The *in vitro* translated (IVT) protein from the cDNA clone has an indistinguishable size to the protein in the NIH3T3 extract, suggesting that this cDNA clone encodes a full length protein of mSir2 $\alpha$  (right panel, Figure 2B). The 120kD band was not detected in immunoprecipitates incubated  
20 with rabbit IgG alone or the *in vitro* translation mixture containing no RNA (Figure 2B). An asterisk indicates IgG in immunoprecipitates.

In the mSir2 $\alpha$ , the greatest homology to ySir2p resides in the middle or core domain of the protein (Figure 2C). The amino acid identity of the mSir2 $\alpha$  core domain to ySir2p is 45.9% and the N-terminal region of the mSir2 $\alpha$  protein also shows weak  
25 homology to ySir2p (13.4% identity). The mSir2 $\alpha$  has a longer C-terminal region than ySir2p, but no significant homology to other proteins in this region. The highly conserved core domains of yeast and mouse *SIR2* family members have homology to the

*Salmonella typhimurium* CobB protein (Tsang, A. W., *et al.*, *J. Biol. Chem.* 273: 31788-31794 (1998)) (20.3% identity in the core domain (Figure 2D). Asterisks denote a motif of putative NAD binding clefts conserved in known mono-ADP-ribosyltransferases. (Figure 2C).

5 In yeast, immunostaining revealed Sir2p localization in the nucleolus and telomeres (Gotta, M., *et al.*, *Embo J.* 16: 3243-3255 (1997)). Immunostaining of mouse NIH3T3 cells with a polyclonal antibody raised against the N-terminal fragment of mSir2 $\alpha$  localized mSir2 $\alpha$  in the nucleus (Figure 3A). DAPI-dense regions in nuclei, which correspond to mouse centromeres, were excluded (Figure 3B and C). This  
10 staining pattern was observed throughout the cell cycle except on mitotic chromosomes, which lacked staining (data not shown). Surprisingly, mSir2 $\alpha$  was not localized to nucleoli, telomeres, or centromeres by co-immunofluorescence (Figure 3, D-F, G-I, J-L) indicating that is not associated with the most highly repeated DNA in the mouse genome.

#### 15 **Yeast and mouse Sir2 proteins are novel nuclear mono-ADP-ribosyltransferases**

The ADP-ribosylase activity of bacterial recombinant full-length proteins of ySir2p and mSir2 $\alpha$  was determined in *in vitro* assays (Figure 4A). Calf thymus histones were used as substrates and  $^{32}$ P-labeled nicotinamide adenine dinucleotide (NAD) as a donor of ADP-ribose. Reaction products were visualized by SDS-PAGE.

20 The recombinant rySir2p and r-mSir2 $\alpha$  proteins transferred radioactive ADP-ribose to H2B and H3, but not to H1, H2A and H4 (Figure 4B, C). The Sir2 proteins modified H2B and H3 in a dose-dependent manner, the ADP-ribosylase activity did not reside in the mSir2 $\alpha$ -specific N-terminal fragment N-ter (Figure 4D). As shown in the upper panel of Figure 4D, 5, 10, and 15  $\mu$ g of recombinant full-length mSir2 $\alpha$  (r-mSir2 $\alpha$ ) or its  
25 N-terminal fragment (N-ter) or control eluate (pET) were added to the reaction with histone H2B. Only the full-length protein showed the dose-dependent modification. As shown in the lower panel of Figure 4D, 100, 250, 500, and 1000 ng of recombinant

full-length ySir2p (r-ySir2p) were added to the reaction with histone H3. ADP-ribosylase activity was not observed when histone proteins were incubated with control pET alone (Figure 4B).

To gain further evidence that this modification is due to ADP-ribose, the ySir2p-  
 5 or mSir2 $\alpha$ -modified H2B and H3 with snake venom phosphodiesterase (SVP) that can digest the phosphodiester bond in the ADP-ribose moiety (Figure 4C, E). The radiolabeled P<sup>32</sup> (P\*) is located at  $\alpha$  position and the phosphodiester bond between radiolabeled and nonlabeled phosphates can be digested by snake venom phosphodiesterase (SVP), resulting in the removal of the radiolabel from  
 10 ADP-ribosylated proteins (Figure 4E). The radiolabel could be substantially removed from H2B and H3 by SVP (Figure 4E). The label remaining in the histones may be due to the forward reaction by Sir2 enzymes and NAD during this treatment.

Histone products of Sir2 modification of unique molecular weights were observed in this assay, suggesting that this modification may not be poly-ADP-ribosylation, but  
 15 mono-ADP-ribosylation. The sensitivity of the ADP-ribosylase activity of mSir2 $\alpha$  to poly ADP-ribosylation inhibitors (3-aminobenzamide and benzamide) and mono-ADP-ribosylation inhibitors (novobiocin and coumermycin A1) was evaluated. (Figure 4F).. 3-aminobenzamide and benzamide did not inhibit the ADP-ribosylation activity of the mSir2 $\alpha$  protein at concentrations of 40, 200 and 1000  $\mu$ M (Figure 4F, lanes 2 to 7)  
 20 whereas novobiocin and coumermycin A1 strongly inhibited ADP-ribosylation at concentrations of 200 $\mu$ M and 40 $\mu$ M, respectively (Figure 4F, lanes 9 and 12), consistent with their reported IC<sub>50</sub> concentrations of 370 and 27  $\mu$ M. ySir2p showed the same sensitivity to those inhibitors (data not shown). Therefore, the sensitivity of the Sir2 enzymatic activities to those inhibitors demonstrates that these proteins are of the  
 25 mono-ADP-ribosyltransferase class.

ADP-ribosyl-transferase activity could be detected for the endogenous mSir2 $\alpha$  immunoprecipitated from NIH3T3 whole cell extracts (Figure 4G). The endogenous mSir2 $\alpha$  was immunoprecipitated from 300 $\mu$ g of the NIH3T3 whole cell extract with an



anti-mSir2 $\alpha$  polyclonal antibody and the immune complex on Protein A-Sepharose beads was incubated in the reaction buffer containing  $^{32}\text{P}$ -labeled NAD and histone H1 or H2B.

### ADP-ribosyltransferase and NAD hydrolase activities of yeast and mouse Sir2 are

#### 5 specifically triggered by acetylated H3 and H4 N-terminal tail peptides

Extended N-terminal tails of H3 and H4 are known to be important for the formation of Sir complex-mediated heterochromatin structure in yeast (Hecht, A., *et al.*, *Cell* 80: 583-592 (1995)) and are targets for several different types of histone modification, such as acetylation, phosphorylation, and methylation (Hansen, J.C., *et al.*, 10 *Biochemistry* 37: 17637-17641 (1998)). The residues 1-20 of H3 are conserved through evolution and lysines 9 and 14 which are acetylated in the diacetylated peptide are indicated by asterisks in Figure 5A. The residues 1-20 of monoacetylated H4 peptide originate from the human sequence and acetylated residues are indicated by asterisks Figure 5A. Tetra-acetylated H4 peptide corresponds to the *Tetrahymena* sequence 15 where all lysines are acetylated. The N-terminal tails of histones were evaluated as targets for ADP-ribosylation by Sir2 proteins.

Two synthetic peptides corresponding to the first 20 amino acids of H3 either unacetylated or diacetylated (on lysines 9 and 14) (Figure 5A) were evaluated to determine whether yeast and mouse Sir2 proteins could modify these peptides.

20 Reactions were carried out as above and analyzed by thin layer chromatography. Both the yeast and mouse Sir2 modified only the diacetylated H3 tail peptide (Figure 5B). The indicated spot (bracket, Figure 5B) was identified as the modified peptide because it migrated just below the ninhydrin-positive peptide spot on the chromatogram. Further, NAD hydrolase activity of both ySir2p and mSir2 $\alpha$  was stimulated in the presence of the 25 diacetylated peptide, indicated by the arrowhead in Figure 5B. Since Lys9 and Lys14 of H3 are acetylated *in vivo*, this preferential utilization of the acetylated H3 peptide strongly suggests that the tail of H3 is a biologically relevant substrate for Sir2 proteins.

The extended N-terminal tail of histone H4 has four lysines at positions 5, 8, 12, and 16. (Figure 5A). Mutational studies show the importance of these residues in silencing *in vivo*, and Lys5, 8, and 16 are hypoacetylated in yeast chromatin at HM loci. In mammalian cells, Lys16 of H4 is highly acetylated. H4 peptides (residues 1-20), which were acetylated singly at each of these lysines (5, 8, 12 or 16), were synthesized and evaluated for mSir2 $\alpha$  stimulated ADP-ribosyltransferase and NAD hydrolase activities (Figure 5C). A striking specificity in the acetylation pattern of the H4 tail was required to stimulate Sir2 activity. Both ADP-ribosylation and NAD hydrolysis were stimulated by the Lys16Ac peptide, but were affected weakly by the Lys5-, 8-, 12-, and tetra-acetylated peptides (Figure 5C).

#### **Highly conserved amino acid residues in the core domain are essential for the mono-ADP-ribosylation activity of mSir2 $\alpha$**

To analyze the mono-ADP-ribosylation activity more precisely, ten highly conserved amino acid residues in the core domain of mSir2 $\alpha$  were changed to alanine (Figure 6A), mutant recombinant Sir2 proteins produced *E. coli*, and evaluated for ADP-ribosylation activities on H2B and H3 *in vitro*. Protocols for the production of mutant nucleic acid constructs and proteins are well known to the skilled artisan. (See, for example, Ausubel *et al.*, "Current Protocols in Molecular Biology", John Wiley & Sons, NY, NY (1999)).

Aliquots of recombinant protein (10 $\mu$ g) of the wild type recombinant mSir2 $\alpha$  (WT), the pET control pET, and the mutant proteins (G253A, G255A, S257A, I262A, F265A, R266A, G270A, P285A, T336A, H355A) were incubated with H2B or H3. Western blot analysis showed that the amount of each full-length protein used was comparable (Figure 5B, r-mSir $\alpha$ ). All of the mutations of highly conserved residues dramatically affected the mono-ADP-ribosylation activity of mSir2 $\alpha$  (Figure 6B). The results were similar using H2B or H3.

-78-

Eight mutants (G253A, G255A, S257A, I262A, F265A, G270A, T336A, H355A) completely abolished the activity and the two mutants R266A and P285A showed some residual activities compared to the wild type activity (18% for R266A and 5% for P285A, see Figure 6C). The radioactivity of H2B modified by the wild type is assigned to 100% and other data were normalized according to the wild type activity and blots quantitated by Phosphorimaging. The averages and the standard deviations were calculated from two independent experiments. In the core domain of ySir2p, mutations on highly conserved residues also affected the mono-ADP-ribosylation activity. Therefore, the core domain of Sir2 proteins contain the catalytic activity of mono-ADP-ribosylation and the evolutionarily conserved residues in the core domain are essential for this activity.

### **The mSir2 $\alpha$ core domain conveys transcriptional repressive activity in mouse cells**

The *in vivo* function of the mSir2 $\alpha$  mono-ADP-ribosylation activity, was evaluated by constructing expression vectors of wild-type (WT) and each of the mutant core domains (G253A, G255A, S257A, I262A, F265A, R266A, G270A, P285A, T336A, H355A) fused to the yeast Gal4 DNA binding domain (DBD) and transfecting the constructed NIH3T3 cells with a luciferase reporter plasmid containing four upstream Gal4 binding sites.

NIH3T3 cells  $10^6$  were transfected with 4 $\mu$ g of effector plasmids which fuse Sir2 sequences to the DBD, 1 $\mu$ g of a reporter plasmid with luciferase expression driven by GAL4 DNA-binding sites and 1.5 $\mu$ g of SV40 promoter-driven  $\beta$ -galactosidase gene to normalize the transfection efficiency. All luciferase activities after normalization were standardized to DBD alone. The averages and the standard deviations were calculated from three independent transfection experiments. The wild-type core domain was able to repress transcription four-fold, compared to the activity of the DBD only, which is known to activate transcription approximately two-fold in mammalian cells (Figure 6D). The DBD-fused full-length ySir2p also showed similar repressive activity in

NIH3T3 cells, suggesting that the shared mono-ADP-ribosylation activity can be involved in transcriptional repressive activity. All of the core domain mutants except for P285A reduced transcriptional repressive activity (Figure 6D). One explanation for this discrepancy is that the P285A change does not inhibit the catalytic domain but causes

5 the recombinant protein to be misfolded in *E. coli*. Another protein that binds to mSir2 $\alpha$  can rescue such a mutant in mammalian cells.

### Sir2p is an NAD-dependent deacetylase for histone H3

The experiments employed purified recombinant Sir2p in a reaction with NAD and a peptide of the histone H3 amino-terminal tail (residues 1-20) di-acetylated at

10 lysines 9 and 14. These lysines are hypoacetylated in silenced chromatin and mutations at these positions to glycine greatly reduce silencing *in vivo* (Thompson, J. S., *et al.*, *Nature* 369: 245-247 (1994)). The products of a reaction containing 5  $\mu$ g of recombinant yeast Sir2p (79 pmoles) (Figure 8a), 10 $\mu$ g of the H3 peptide (4.2 nmoles) increasing concentrations of NAD were analyzed s by high pressure liquid

15 chromatography (HPLC).

As shown in Figure 8b, the histone peptide reacted in the absence of NAD gave rise to two peaks (3 and 5) which were analyzed by electron-spray mass spectroscopy (Figures 9a and 9d) and correspond to monomer (MW2370) and dimer (MW4740) peptide, the latter likely due to oxidation of the peptide at the carboxyl cysteine residue.

20 The same species were observed in reactions with a control bacterial preparation ("pET" in Figure 8a) in the presence of NAD (not shown).

The addition of NAD to the reaction containing Sir2p gave rise to three additional peaks (1, 2, and 4), as well as an alteration in peak 3 (Figures 8c, 8d, 8e and 8f), which were also analyzed by electron-spray mass spectroscopy. Strikingly, these

25 peaks did not correspond to ADP-ribosylated species, but rather to deacetylated species of peptide (see Figure 8g). Peak 4 corresponded to the singly-deacetylated dimer (MW 4698) (Figure 9c), peak 3 now also contained the doubly-deacetylated dimer (MW4656)

(Figure 9b). Because of their lower abundance, peak 1 and peak 2 were analyzed separately by matrix assisted laser desorption/ionization (MALDI) mass spectroscopy and peak 2 was found to correspond to the triply deacetylated dimer (MW4614) and peak 1 to the singly-deacetylated monomer (MW2328) (not shown). The relative areas under peaks 1, 2, and 4 was quantitated and at least 27% of the input peptide was deacetylated by Sir2p. The approximate  $K_m$  of this deacetylation reaction for NAD was about 100 $\mu$ M, at which the reaction proceeded to about 50% of the maximal level observed at higher concentrations of the cofactor.

To analyze these reaction products further, peak 4, the singly deacetylated dimer, along with the input peak 5 were subjected to N-terminal protein sequencing by Edmann degradation (Figure 10). The only differences between the input and reacted peaks occurred at lysines 9 and 14. Approximately 23-27% of the acetyl lysines at each position were deacetylated by Sir2p in the presence of NAD, in agreement with the calculated efficiency of the reaction and the mass spectroscopy data above. The unacetylated lysine 18 of peaks 4 and 5 is also shown for comparison. Thus, Sir2p is an NAD-dependent histone deacetylase which can deacetylate either lysine 9 or 14 of the H3 N-terminus.

### Effects of inhibitors on deacetylation and putative ADP-ribosylation

The effects of a potent inhibitor of histone deacetylases, trichostatin A (TSA) (Yoshida, M., *et al.*, *A. J. Biol. Chem.* 265: 17174-17179 (1990)) was evaluated. TSA has been shown to act on constitutive heterochromatin *in vivo*, since it disrupts centromeric heterochromatin in *S. pombe* (Ekwall, K., *et al.*, *Cell* 91: 1021-1032 (1997)). As shown in Figures 11a and 11b, TSA was totally incapable of inhibiting deacetylation, indicating that Sir2p is fundamentally different from the class of TSA-sensitive histone deacetylases, including Rpd3 (Taunton, J., *et al.*, *Science* 272: 408-411 (1996)).

Sir2 proteins have been proposed to transfer a single ADP-ribose from NAD to protein (Frye, R. A., *Biochem. Biophys. Res. Commun.* 260: 273-279 (1999)).

Consistent with this, we found that  $^{32}\text{P}$  was transferred from NAD to intact histone H3 (Figure 11c) or to the H3 peptide (Figure 11d). Transfer of label to the peptide was assayed using thin layer chromatography (TLC), which revealed not only this transfer but a substantial amount of NAD hydrolysis.

- 5        The effects of a known inhibitor of mono-ADP-ribosyltransferases, coumermycin A1 (Banasik, M., *et al.*, *Mol. Cell. Biochem.* 138: 185-187 (1994)) was evaluated. Both ADP-ribosylation (Figures 11c and 11d) and NAD hydrolysis (Figure 11d) were inhibited by this drug at concentrations known to inhibit other mono-ADP-ribosyltransferases. Strikingly, coumermycin A1 was *not* capable of inhibiting
- 10    deacetylation of the H3 tail by Sir2p (Figure 11e). Thus, the ADP-ribosyltransferase and NAD hydrolase reactions are fundamentally distinct from this NAD-dependent deacetylation reaction. These two separable enzymatic activities of Sir2p may play distinct roles *in vivo*.

#### **Mouse Sir2p also catalyzes the deacetylation of the histone H3 tail**

- 15        A mouse *Sir2* homolog was identified in the EST database and cloned the full length cDNA, termed mSir2 $\alpha$  (Figure 12a). The conserved region of this protein resembles yeast Sir2p more closely than do other mouse Sir2 proteins (Figure 12b). To determine whether the mouse Sir2p homolog mSir2 $\alpha$  would catalyze this deacetylation reaction, we incubated purified recombinant mSir2 $\alpha$  with the di-acetylated H3 peptide
- 20    and analyzed the reaction products by HPLC (Figure 12c). The murine protein gave rise to the same array of products with a similar yield as the yeast enzyme, indicating that this deacetylation reaction is conserved from yeast to mammals.

#### **NADH, NADP, and NADPH do not activate deacetylation by Sir2p**

- 25        While there are literally hundreds of NAD-linked dehydrogenases that use NAD and NADH in oxidation/reduction reactions in cells, for example, glyceraldehyde-3-phosphate dehydrogenase, the requirement for NAD to drive any other enzymatic

reaction is novel. This suggested that Sir2 proteins might be sensors of the energy or oxidation state of cells that transduces this status to the organization of chromatin structure. Catabolic reactions in cells, such as substrate level and oxidative phosphorylation in the utilization of glucose, are oxidative and use NAD to produce NADH. Biosynthetic reactions typically are reductive and use NADPH to produce NADP. It was thus of interest to determine whether NADH, NADP, and NADPH would function in the deacetylation reaction.

As shown in Figure 13, NADP and NADPH did not function significantly, while NADH functioned weakly in the deacetylation of the H3 peptide by Sir2p. In fact, the small amount of deacetylated products in the NADH reaction can reasonably be attributed to a low level of oxidation of the NADH preparation. Also, neither NADH nor NADP significantly inhibited the deacetylase activity in a reaction with NAD (not shown). This remarkable specificity of Sir2p for NAD but not the other dinucleotides may allow Sir2 proteins to sense the energy or oxidation status of cells to link histone deacetylation and chromatin silencing to the metabolic rate.

### **Histone deacetylase defective Sir2p mutants show defects in silencing, recombination suppression and life span**

The present invention shows that mutations in Sir2 proteins reduce or eliminate the enzymatic activity of the protein affect these various functions, indicating that the histone deacetylase activity of Sir2p is important *in vivo*.

A set of mutations in highly conserved residues of the core domain of *SIR2* were constructed by site-directed mutagenesis (Figure 17a) and cloned into vectors, along with the wild type, to allow expression of the recombinant proteins in *E. coli* or expression of single-copy genes from the native *SIR2* promoter in *S. cerevisiae*. These 6Xhis tagged proteins were purified from *E. coli* by a Ni-NTA column (Figure 17b) and analyzed for the NAD-dependent histone H3 deacetylase activity in an assay with a di-acetylated H3 peptide (residues 1-20 acetylated on Lys9 and Lys14) and 1mM NAD. HPLC separation of the reaction products yields five peaks of which 1, 2, a portion of 3

and 4 are deacetylated species of peptide (Figure 15). In this deacetylase assay, mutants 345 and 347 were inactive, mutant 261 showed 17% of wild type activity, mutants 270 and 271 showed 80% and 36% wild type activity, respectively, and mutant 275 showed 67% wild type activity.

5        The Sir 2 mutant proteins were also analyzed for ADP-ribosyltransferase activity using the histone H3 substrate, (Figure 17c). Mutants 345 and 347 were completely inactive, mutants 261, 270, and 271 displayed very weak activity, 4%, 7%, and 8%, respectively, and mutant 275 was about as active as wild type. The same pattern of activities was observed using a peptide of the N-terminal tail of histone H3 (not shown).

10        To examine the functions of these mutants *in vivo*, we studied yeast indicator strains in which the endogenous *SIR2* had been deleted and the wild type and mutant *SIR2* genes integrated back into the genome. All strains used are isogenic derivatives of W303R (Kaeberlein, M., *et al.*, *Genes Dev.* 13: 2570-2580 (1999)) and all results are summarized in Figure 18. The mutant proteins were visualized by Western blot and  
15        found to be expressed at approximately the same level as wild type, with a small reduction in the level of mutant 261 and perhaps 275, and 345 (Figure 16a).

      Silencing of the *HML $\alpha$*  and *HMRA* mating loci was assayed by mating with a haploid strain of opposite mating type and monitoring the appearance of diploids on selective media. In this assay (Figure 16b), mutants 261, 270, and 271 were mating  
20        proficient, indicating that silencing was essentially intact, and mutants 345, 347, and, surprisingly, 275 were defective. Telomere silencing was determined by repression of the telomere-positioned *URA3* gene, which gives rise to growth on media containing 5-fluoro-orotic acid (FOA). Serial dilution of wild type and mutant strains on FOA plates indicated that mutant 261 silenced about as well as wild type, 270 showed partial  
25        silencing, and 271, 275, and 345 were defective (Figure 16c). Silencing in the rDNA was determined by a serial dilution onto adenine-lacking media of strains with *ADE2* inserted into the rDNA (Kaeberlein, M., *et al.*, *Genes Dev.* 13: 2570-2580 (1999)). To facilitate detectable differences in rDNA silencing, a *rpd3 $\Delta$*  strain was used which



displays enhanced silencing (Smith, J. S., *et al.*, *Mol. Cell Biol.* 19: 3184-3197 (1999)). By this assay mutants 261 and 270 silenced about as well as the wild type, mutant 271 showed weak silencing, and mutants 275, and 345 were totally defective (Figure 16d).

The data indicate a general, although not strictly quantitative, correlation  
 5 between deacetylase activity *in vitro* and silencing *in vivo* (except for 275, see below). Mutant 345 is inactive for deacetylase activity *in vitro* and silencing *in vivo*. Mutants 261, 270, and 271, have intermediate levels of deacetylase activities *in vitro* and intermediate effects on silencing *in vivo*. Mutant 261 is proficient in all silencing assays, but does show a defect in a more sensitive silencing assay of life span, below. Mutant  
 10 270 is proficient in HM and rDNA silencing but intermediate in silencing at telomeres, and 271 is proficient in HM silencing, but intermediate to weak in silencing at telomeres and rDNA. An intermediate levels of deacetylase activity can are sufficient to mediate silencing, but the specific level of silencing may be modulated by other variables affected by these mutations, such as the interaction of Sir2p with partner proteins. The  
 15 lack of silencing activity in mutant 275 was unexpected and may reflect an inability of that mutant protein to localize properly in the nucleus or to interact with important partners *in vivo*.

Replicative life span of mother cells is due in part to the accumulation of extrachromosomal rDNA circles (Sinclair, D. A., *et al.*, *Science* 277: 1313-1316  
 20 (1997));(Sinclair, D. A., *et al.*, *Cell* 91: 1-20 (1997)) that arise by homologous recombination (Park, P. U., *et al.*, *Mol. Cell. Biol.* 19: 3848-3856 (1999)) in the tandem array of rDNA repeats on chromosome XII. This recombination is suppressed 5-10 fold by *SIR2* (Gottlieb, S., *et al.*, *Cell* 56: 771-776 (1989)), allowing cells to enjoy their normal life span. To measure recombination frequencies, wild type and Sir2 mutant  
 25 strains containing the *ADE2* gene inserted into the rDNA were plated on YPD media. This media is limiting in adenine, causing *ade2-* colonies to accumulate a red pigment. Half-red/white sector colonies indicate *ADE2* loss in the first generation after plating, and the frequency of these colonies compared to Ade<sup>+</sup> colonies is a direct measure of the recombination rate in the rDNA. By this assay, *SIR2* suppressed recombination about

12-fold compared to the *SIR2* deletion strain (Figure 17a). Mutants 261 and 270, showed a high degree of suppression in the range of wild type, mutant 271 showed partial suppression, and mutants 275, and 345 were as defective as the *SIR2* deletion. Thus, the activities of these mutants in this recombination assay are similar to their activities in the

5 rDNA silencing assay, above.

Previous findings show that the short life span of *SIR2* deletion mutants in strain W303R is a composite of a failure to suppress rDNA recombination and also a failure to repress transcription of the copies of mating type information at *HM* loci yielding the *a/α* cell type (Kaeberlein, M., *et al.*, *Genes Dev.* 13: 2570-2580 (1999)). Expression of

10 *a/α* leads to a higher rate of recombination in the rDNA. The life spans of representative mutants were first determined in strains in which *HMLα* was deleted to avoid any contribution of the *a/α* cell type if weak derepression occurred at those loci. The *sir2* deletion shortened life span about 50% compared to wild type, as expected (Figure 17b). Mutants 261 and 270 complemented the *sir2* deletion to give a wild type life span and

15 mutant 275 was completely defective in complementing the life span defect, consistent with the activities of these mutants in the recombination assay.

Life span determination in wild type HM strains is a very sensitive known assay for *Sir2* activity *in vivo*, i.e. in this assay even *SIR2/SIR2* heterozygous diploids show a pronounced defect (Kaeberlein, M., *et al.*, *Genes Dev.* 13: 2570-2580 (1999)). Thus, the

20 life spans of *SIR2* mutants were also determined in otherwise wild type haploid strains (Figure 17c). Now a partial defect *was* observed in the 261 and 270 mutants, which can be attributed to a slight defect in the maintenance of repression at *HM* loci. Mutant 275 was again defective. Thus, with this sensitive assay, the 261 and 270 *SIR2* mutants show defects, which parallel their enzymatic defects *in vitro*. Mutant 275 is exceptional,

25 as in the above assays.

Previous studies indicate that the acetylation state of lysines in the amino-terminal tails of histones H3 and H4 is crucial for silencing *in vivo* (Braunstein, M., *et al.*, *Genes Dev* 7: 592-604 (1993); (Braunstein, M., *et al.*, *Mol. Cell. Biol.* 16: 4349-4356 (1996);

(Turner, B. M., *et al.*, *Cell* 69: 408-411 (1992)). Lysines 9 and 14 of H3 and lysines 5, 8, and 16 of H4 are hypoacetylated in silenced chromatin and return to their acetylated state in SIR mutants (Braunstein, M., *et al.*, *Genes Dev* 7: 592-604 (1993); (Braunstein, M., *et al.*, *Mol. Cell. Biol.* 16: 4349-4356 (1996)). Moreover, mutations in the lysines of the H3 tail, especially to uncharged residues, cause a loss of silencing (Thompson, J. S., *et al.*, *Nature* 369: 245-247 (1994)). These data show that *Sir2* mutations which eliminate deacetylase activity show silencing defects *in vivo*.

Taken together these findings suggest that the NAD-dependent histone deacetylase activity of Sir2p sufficiently explains the *SIR2* functions of silencing, suppression of rDNA recombination, and life span extension *in vivo*. H3 and H4 are acetylated as a prerequisite to their recognition by chromatin assembly factors (Ma, X. J., *et al.*, *Proc. Nat. Acad. Sci.* 95: 6693-6698 (1998)). Sir2p can be a target to specific genomic sites and initiates silencing by deacetylating H3 and, most likely, H4. It is possible that the putative ADP-ribosyltransferase activity of Sir2p also plays some role in these *in vivo* functions.

These data show that histone deacetylation by Sir2p has an absolute requirement for NAD. These data are striking and can couple chromatin silencing to the energy status of cells. Caloric restriction (Weindruch, R. H., *et al.*, *J. Nutrit.* 116: 641-654 (1986)) increases life span in a wide variety of eukaryotic species. This regimen may increase available NAD and trigger the activity of Sir2 proteins, leading to greater silencing and a longer life span. Similarly normal aging can be caused, in part, by a decrease in the activity of Sir2 proteins, perhaps owing to a reduction in available NAD, causing a loss of silencing and the deleterious alteration in the pattern of gene expression.

Mouse Sir2p homolog mSir2 $\alpha$  has histone deacetylase activity that closely resembles yeast Sir2p. The Sir2 proteins described herein and related mammalian Sir2 proteins (Brachmann, C. B., *et al.*, *Genes Dev.* 9: 2888-2902 (1995); (Frye, R. A., *Biochem. Biophys. Res. Commun.* 260: 273-279 (1999)) could play regulated roles in

chromatin structure, which might include changes in genome organization during cellular differentiation and in response to physiological stimuli, including caloric restriction. If aging were due, at least in part, to a loss of silencing, then interventions to increase the activity of Sir2 proteins can slow aging in mammals.

## CLAIMS

What is claimed is:

1. A method of altering the NAD-dependent acetylation status of at least one amino acid residue in a histone protein by altering the activity of a Sir2 protein.
- 5 2. The method of Claim 1, wherein the histone protein is selected from the group consisting of an H2B, H3 or H4 histone protein.
3. The method of Claim 1, wherein the amino acid residue is a lysine amino acid residue.
4. The method of Claim 3, wherein the lysine amino acid residue is lysine 9 and/or  
10 lysine 14 of an H3 histone protein.
5. The method of Claim 3, wherein the lysine amino acid residue is lysine 16 of an H4 histone protein.
6. The method of Claim 1, wherein the alteration in NAD-dependent acetylation status is removal of an acetyl group.
- 15 7. The method of Claim 1, wherein the Sir2 protein is a Sir2 $\alpha$  protein.
8. The method according to Claim 7, wherein the Sir2 $\alpha$  protein has the amino acid sequence selected from the group consisting of SEQ ID NOS: 1, 4, 9, 12, 19 or 26.

9. The method according to Claim 7, wherein the Sir2 $\alpha$  protein is encoded by the nucleic acid sequence of SEQ ID NO: 25.
10. The method of Claim 7, wherein the Sir2 $\alpha$  protein is a mutant Sir2 $\alpha$  protein selected from the group consisting of G253A, G255A, S257A, I262A, F265A, R266A, G270A, P285A, T336A, H355A, Thr-261, Iso-271, Arg-275 or Asn-345.
11. A method of identifying an agent which alters the activity of a Sir2 protein by assessing the ability of the agent to alter the NAD-dependent acetylation status of at least one amino acid in a histone protein, comprising the steps of:
- (a) combining the histone protein, the Sir2 protein, NAD compound and the agent to be tested, thereby producing a combination;
  - (b) detecting the NAD-dependent acetylation status of an amino acid in the histone protein in the combination; and
  - (c) comparing the NAD-dependent acetylation status of an amino acid in the histone protein in the combination with the NAD-dependent acetylation status of the amino acid in the histone protein in the absence of the agent to be tested,
- wherein a difference in the NAD-dependent acetylation status of the amino acid of the histone protein in the presence of the agent as compared with the absence of the agent indicates that the agent alters the NAD-dependent acetylation status of at least one amino acid of the histone protein.
12. The method of Claim 11, wherein the agent is an agonist of the activity of the Sir2 protein.
13. The method of Claim 11, wherein the agent is an antagonist of the activity of the Sir2 protein.

14. The method of Claim 11, wherein the alteration of NAD-dependent acetylation status is removal of an acetyl group.
15. The method of Claim 11, wherein the histone protein is selected from the group consisting of an H2B, H3 or H4 histone protein.
- 5 16. The method of Claim 11 wherein the NAD-dependent acetylation in the histone protein is acetylation of a lysine amino acid residue.
17. The method of Claim 16, wherein the lysine amino acid residue is lysine 9 and/or lysine 14 of an H3 histone protein.
- 10 18. The method of Claim 16, wherein the lysine amino acid residue is lysine 16 of an H4 histone protein.
19. The method of Claim 11, wherein the Sir2 protein is a Sir2 $\alpha$  protein.
20. The method of Claim 11, wherein the detecting step is performed by electron-spray mass spectroscopy.
- 15 21. A method of identifying an agent which alters life span of a cell by assessing the ability of the agent to alter NAD-dependent acetylation status of at least one amino acid in a histone protein, comprising the steps of:
  - (a) combining the histone protein, a Sir2 protein, a NAD compound and the agent to be tested, thereby producing a combination;
  - 20 (b) detecting the NAD-dependent acetylation status of an amino acid in the histone protein in the combination; and

- (c) comparing the NAD-dependent acetylation status of an amino acid in the histone protein in the combination with the acetylation status of the amino acid in the histone protein in the absence of the agent to be tested, wherein a difference in the acetylation status of the amino acid of the histone protein in the presence of the agent as compared with the acetylation status of the amino acid of the histone protein in the absence of the agent indicates that the agent alters the life span of the cell.
22. The method of Claim 21, wherein the histone protein is selected from the group consisting of an H2B, H3 or H4 histone protein.
23. The method of Claim 21, wherein the Sir2 protein is a Sir2 $\alpha$  protein.
24. The method of Claim 21, further comprising administering to a cell the agent identified by the method and assessing the NAD-dependent acetylation status of at least one amino acid in a histone protein of the cell.
25. A method of altering the NAD-dependent acetylation status of at least one amino acid residue in a histone protein comprising combining the histone protein, a Sir2 protein and a NAD compound.
26. The method of Claim 25, wherein the histone protein is selected from the group consisting of an H2B, H3 or H4 histone protein.
27. The method of Claim 25, wherein the Sir2 protein is a Sir2 $\alpha$  protein.
28. A method of identifying an agent which alters mono-ADP-ribosylation of a nuclear protein in a cell, comprising the steps of:
- combining a cell and an agent to be tested;



- b) determining a level of mono-ADP-ribosylation of a nuclear protein in said cell; and
- c) comparing the level determined in step (b) with a level of mono-ADP-ribosylation of the nuclear protein in the absence of the agent to be tested,
- 5 wherein a difference in the level of mono-ADP-ribosylation of the nuclear protein between the presence of the agent and the absence of the agent indicates that the agent alters mono-ADP-ribosylation of the nuclear protein.
29. The method according to Claim 28, wherein the nuclear protein is a histone protein.
- 10 30. The method according to Claim 28, wherein the mono-ADP-ribosylation is performed by a Sir2 protein.
31. The method according to Claim 30, wherein the mono-ADP-ribosylation is performed by a Sir2 $\alpha$  protein.
- 15 32. A method of identifying an agent which alters life span of a cell, comprising the steps of:
- a) combining a cell and an agent to be tested;
- b) determining a level of mono-ADP-ribosylation of a nuclear protein in said cell; and
- c) comparing the level determined in step (b) with a level of mono-ADP-ribosylation of the nuclear protein in the absence of the agent to be tested,
- 20 wherein a difference in the level of mono-ADP-ribosylation of the nuclear protein between the presence of the agent and the absence of the agent indicates that the agent alters the life span of the cell.

33. The method according to Claim 32, wherein the nuclear protein is a histone protein.
34. The method according to Claim 32, wherein the mono-ADP-ribosylation is performed by a Sir2 protein.
- 5 35. The method according to Claim 34, wherein the mono-ADP-ribosylation is performed by a Sir2 $\alpha$  protein.
36. A method of identifying an agent which alters aging of a cell, comprising the steps of:
- 10 a) combining a cell and an agent to be tested;
- b) determining a level of mono-ADP-ribosylation of a nuclear protein in said cell; and
- c) comparing the level determined in step (b) with a level of mono-ADP-ribosylation of the nuclear protein in the absence of the agent to be tested, wherein a difference in the level of mono-ADP-ribosylation of the nuclear protein
- 15 between the presence of the agent and the absence of the agent indicates that the agent alters aging of the cell.
37. The method according to Claim 36, wherein the nuclear protein is a histone protein.
- 20 38. The method according to Claim 36, wherein the mono-ADP-ribosylation is performed by a Sir2 protein.
39. A method of increasing the life span of a cell, comprising administering to the cell an effective amount of an agent which increases mono-ADP-ribosylation of a

nuclear protein, wherein said agent is identified by a method comprising the steps of:

- a) combining a cell and an agent to be tested;
  - b) determining a level of mono-ADP-ribosylation of a nuclear protein in said cell; and
  - c) comparing the level determined in step (b) with a level of mono-ADP-ribosylation of the nuclear protein in the absence of the agent to be tested,
- wherein in the presence of the agent there is an increase in the level of mono-ADP-ribosylation of the nuclear protein.

10 40. The method according to Claim 39, wherein the nuclear protein is a histone protein.

41. The method according to Claim 39, wherein the mono-ADP-ribosylation is performed by a Sir2 protein.

15 42. The method according to Claim 39, wherein the agent is selected from the group consisting of a SIR2 protein, a mono-ADP-ribosyltransferase, an agonist of a SIR2 protein, an agonist of a mono-ADP-ribosyltransferase or combinations thereof.

20 43. A method of decreasing aging of a cell, comprising administering to the cell an effective amount of an agent which increases mono-ADP-ribosylation of a nuclear protein, wherein said agent is identified by a method comprising the steps of:

- a) combining a cell and an agent to be tested;
- b) determining a level of mono-ADP-ribosylation of a nuclear protein in said cell; and
- c) comparing the level determined in step (b) with a level of mono-ADP-ribosylation of the nuclear protein in the absence of the agent to be tested,

wherein in the presence of the agent there is an increase in the level of mono-ADP-ribosylation of the nuclear protein.

44. The method according to Claim 43, wherein the nuclear protein is a histone protein.
- 5 45. The method according to Claim 43, wherein the mono-ADP-ribosylation is performed by a Sir2 protein.
46. A method of increasing the life span of a cell comprising administering to the cell a mono-ADP-ribosyltransferase or an agonist of a mono-ADP-ribosyltransferase in an amount effective to increase the life span of the cell.
- 10 47. The method according to Claim 46, wherein the mono-ADP-ribosyltransferase is a Sir2 protein.
48. The method according to Claim 47, wherein the mono-ADP-ribosyltransferase is a SIR2 $\alpha$  protein.
49. The method according to Claim 47, wherein the SIR2 protein comprises the amino  
15 acid sequence selected from the group consisting of SEQ ID NOS: 1, 4, 9, 12, 19, or 26.
50. The method according to Claim 47, wherein the SIR2 protein is encoded by a nucleic acid sequence of SEQ ID NO: 25.
51. A method of increasing the life span of a cell comprising administering to the cell  
20 an agonist of mono-ADP-ribosylation of histones H2B, H3 or H4 or combinations thereof in an amount effective to increase the life span of the cell.

52. A method of decreasing aging of a cell comprising administering to the cell a mono-ADP-ribosyltransferase or an agonist of a mono-ADP-ribosyltransferase in an amount effective to decrease aging of the cell.
53. A method of decreasing aging of a cell comprising administering to the cell an agonist of mono-ADP-ribosylation of a histone H2B in an amount effective to decrease aging of the cell.
54. A method of inhibiting formation, replication and/or accumulation of rDNA circles in a cell comprising administering to the cell a mono-ADP-ribosyltransferase or an agonist of a mono-ADP-ribosyltransferase in an amount effective to inhibit the formation, replication and/or accumulation of rDNA circles.
55. A method for decreasing recombination between rDNA in a cell comprising administering to the cell a mono-ADP-ribosyltransferase or an agonist of a mono-ADP-ribosyltransferase in an amount effective to decrease recombination between rDNA.
56. An isolated murine Sir2 protein.
57. Isolated murine Sir2 protein of Claim 56, wherein the murine Sir2 protein is a Sir2 $\alpha$  protein.
58. Isolated murine Sir2 $\alpha$  protein of Claim 57, wherein the Sir2 $\alpha$  protein has the amino acid sequence of SEQ ID NO: 26.
59. Isolated murine Sir2 $\alpha$  protein of Claim 57, wherein the Sir2 $\alpha$  protein is encoded by the nucleic sequence of SEQ ID NO: 25.

60. A recombinant host cell comprising an isolated nucleic acid molecule of a murine SIR2 gene operably linked to a regulatory sequence.
  61. An antibody, or an antigen-binding fragment thereof, which selectively binds a murine Sir2 protein or a murine protein.
- 5

0050.1618000

METHODS FOR IDENTIFYING AGENTS WHICH ALTER  
HISTONE PROTEIN ACETYLATION, DECREASE AGING, INCREASE  
LIFESPAN

ABSTRACT OF THE DISCLOSURE

- 5       Methods of identifying agents which alter the NAD-dependent acetylation status  
and mono-ADP-ribosylation of nuclear proteins are disclosed. The methods further  
include identifying agents which alter the life span or aging of a cell or an organism by  
determining the level of NAD-dependent acetylation and/or ADP ribosylation of a  
nuclear protein. The invention also relates to a mammalian Sir2 protein which acetylates  
10 or deacetylates nuclear proteins in a NAD-dependent manner and has mono-ADP-  
ribosyltransferase activity. Host cells producing the Sir2 protein and antibodies to the  
Sir2 protein are also provided.

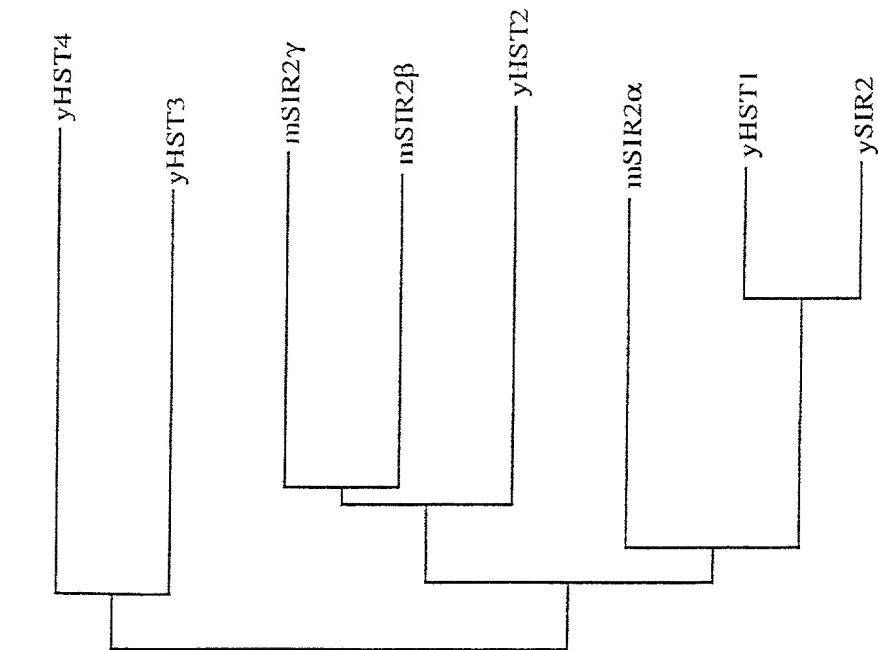


Figure 1A

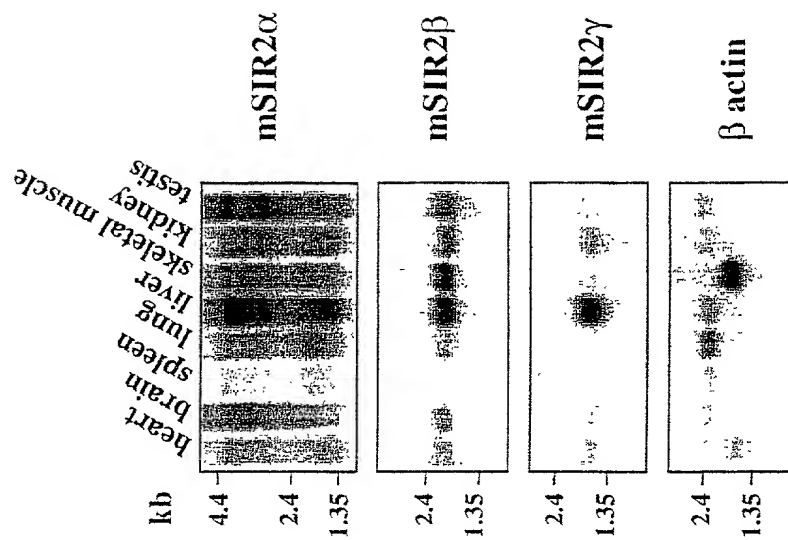


Figure 1B



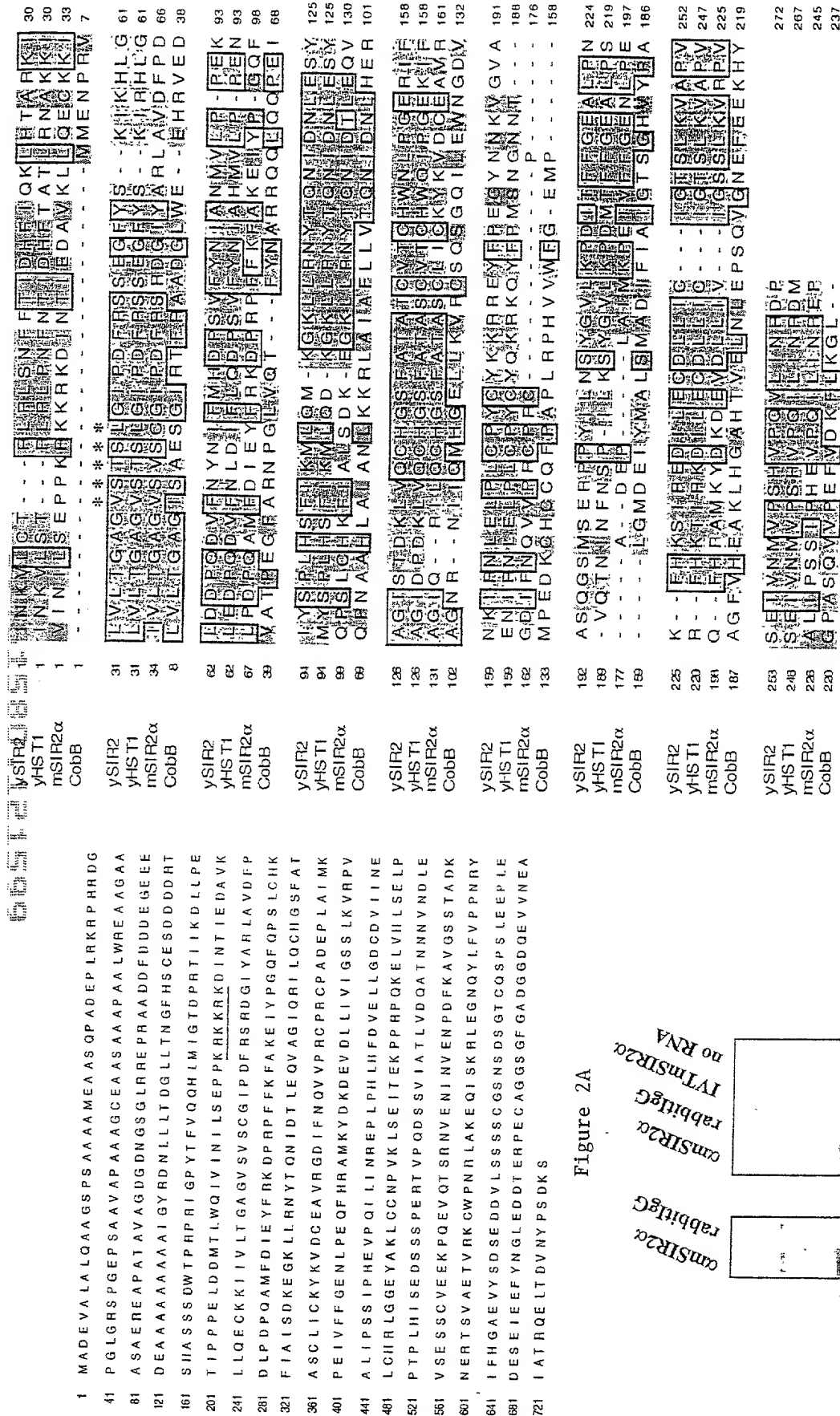


Figure 2C

Figure 2D

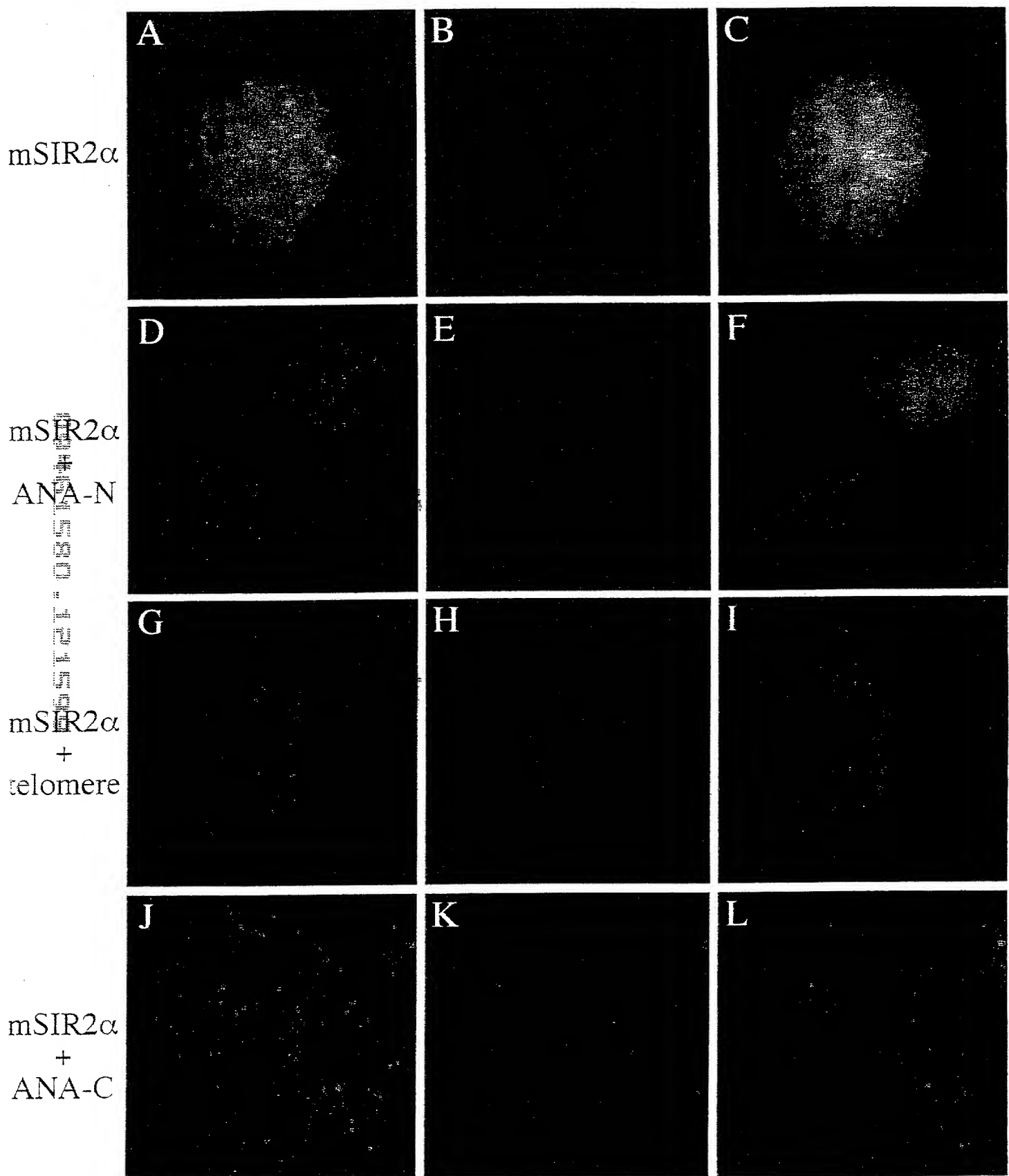


Figure 3

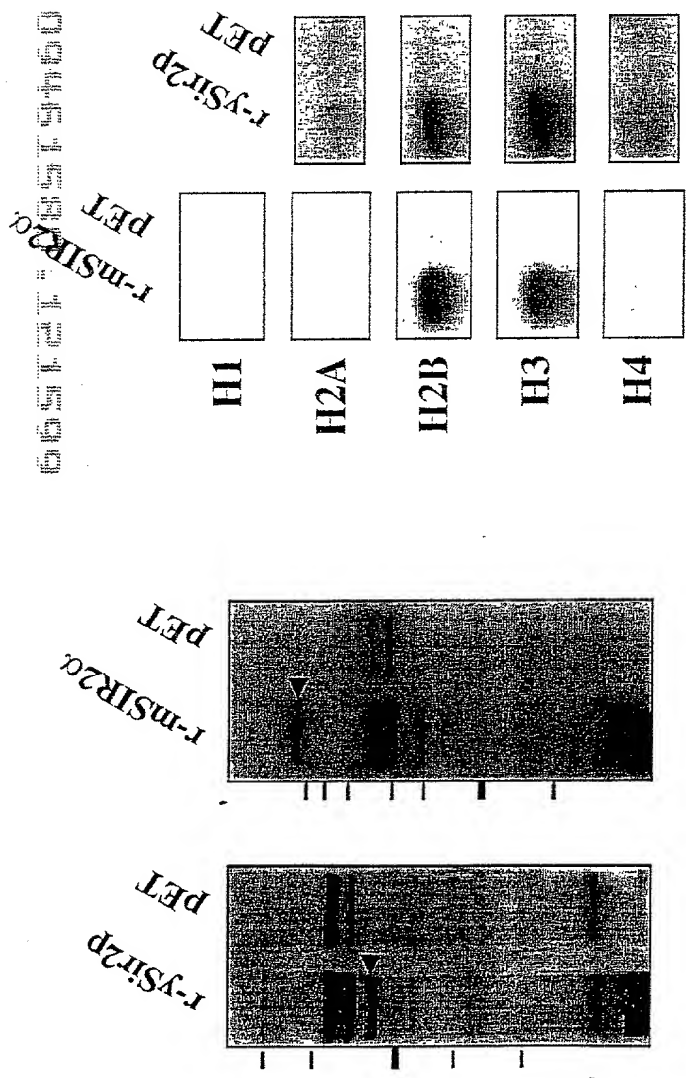


Figure 4A

Figure 4B

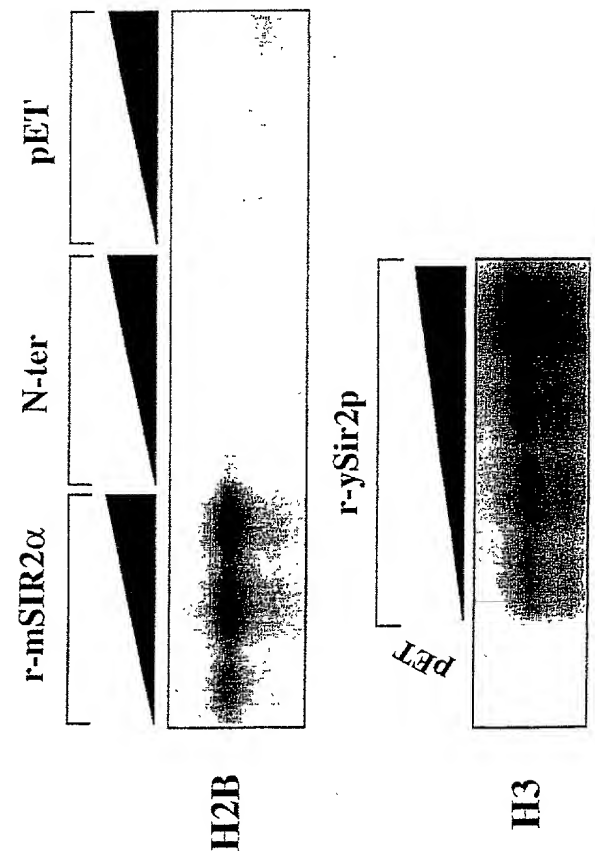


Figure 4D

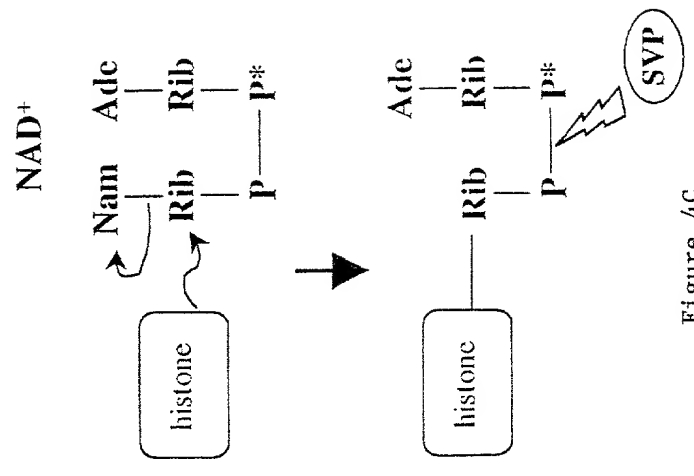


Figure 4C

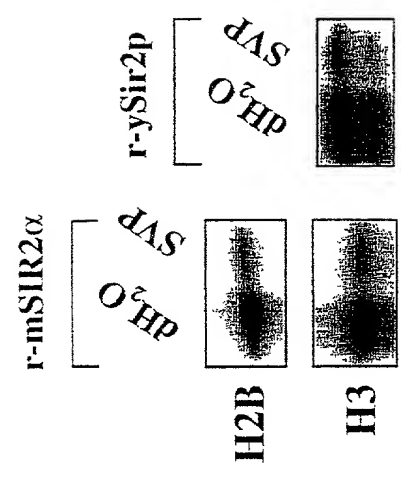


Figure 4E

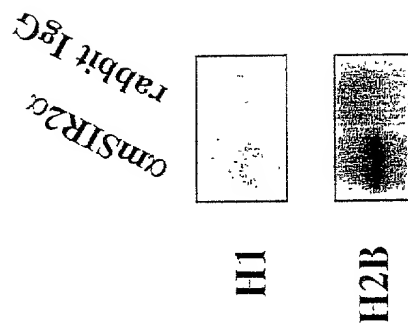


Figure 4G

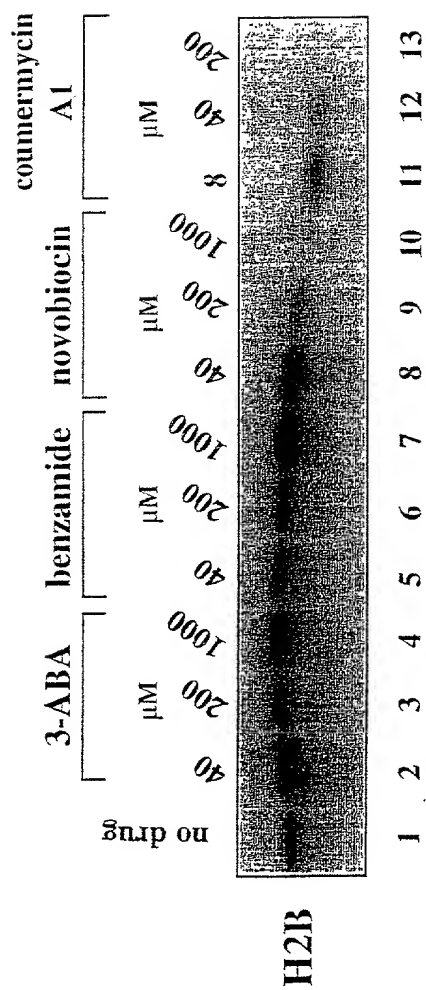


Figure 4F



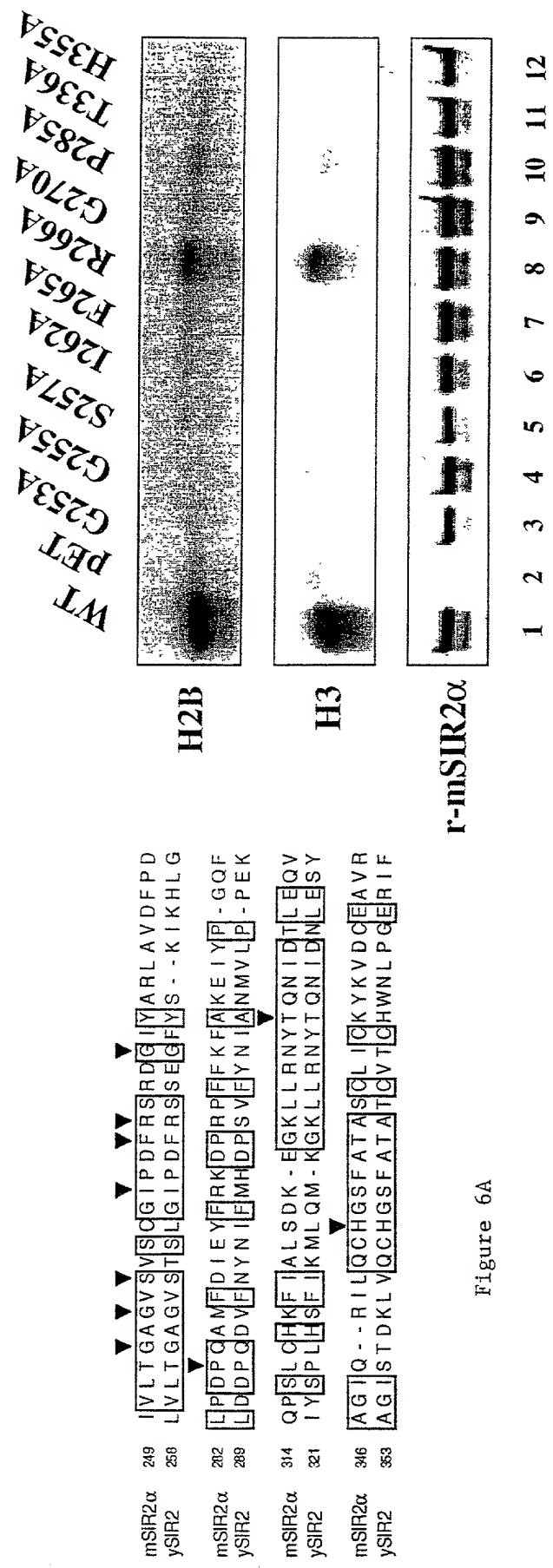


Figure 6B

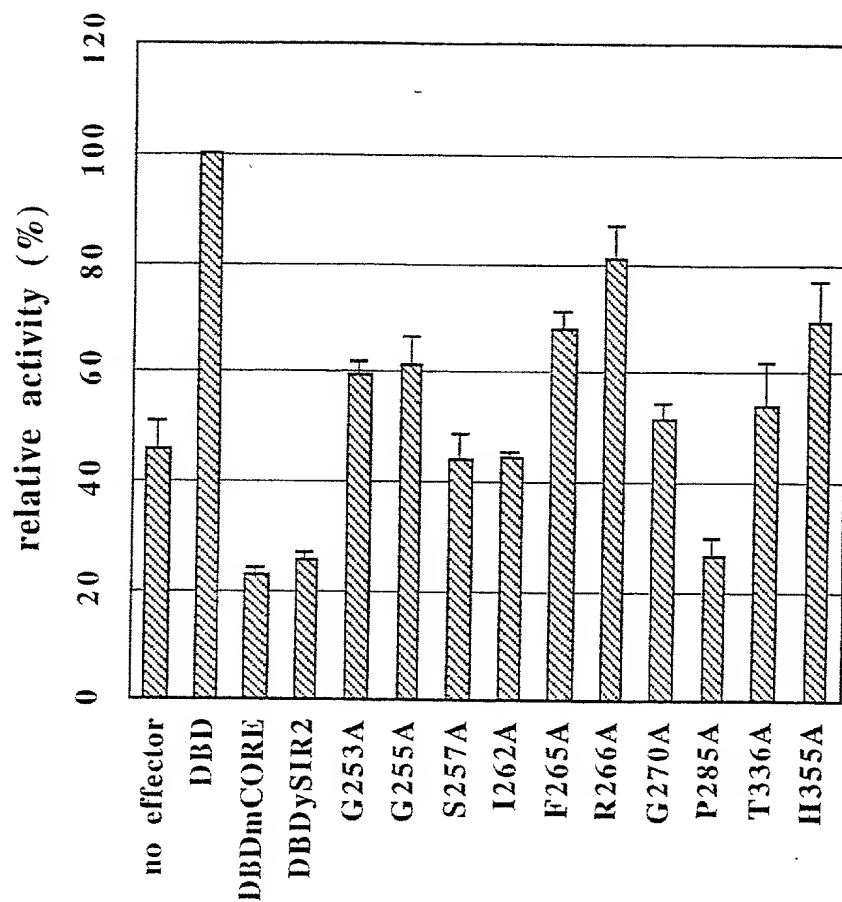


Figure 6D

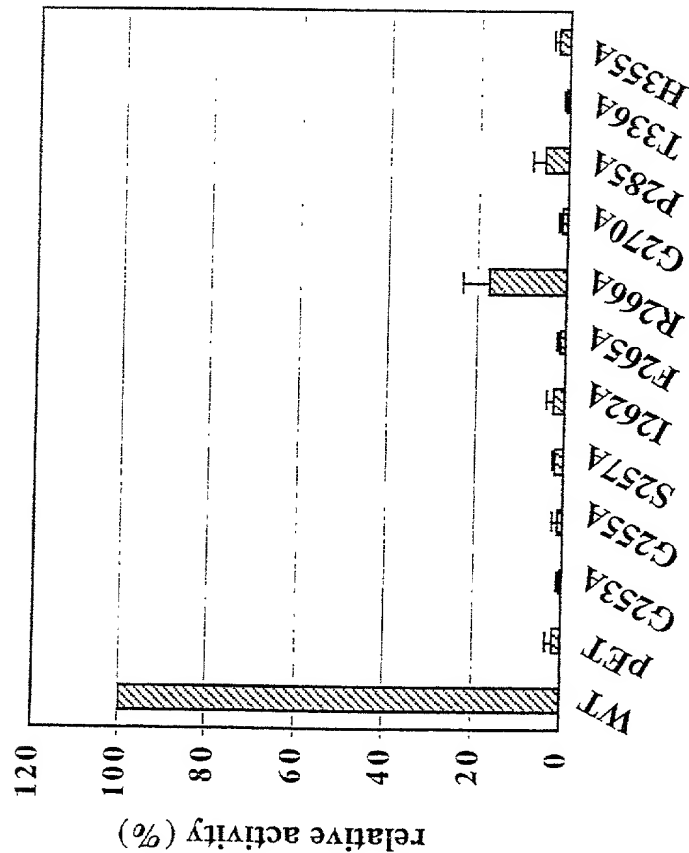


Figure 6C

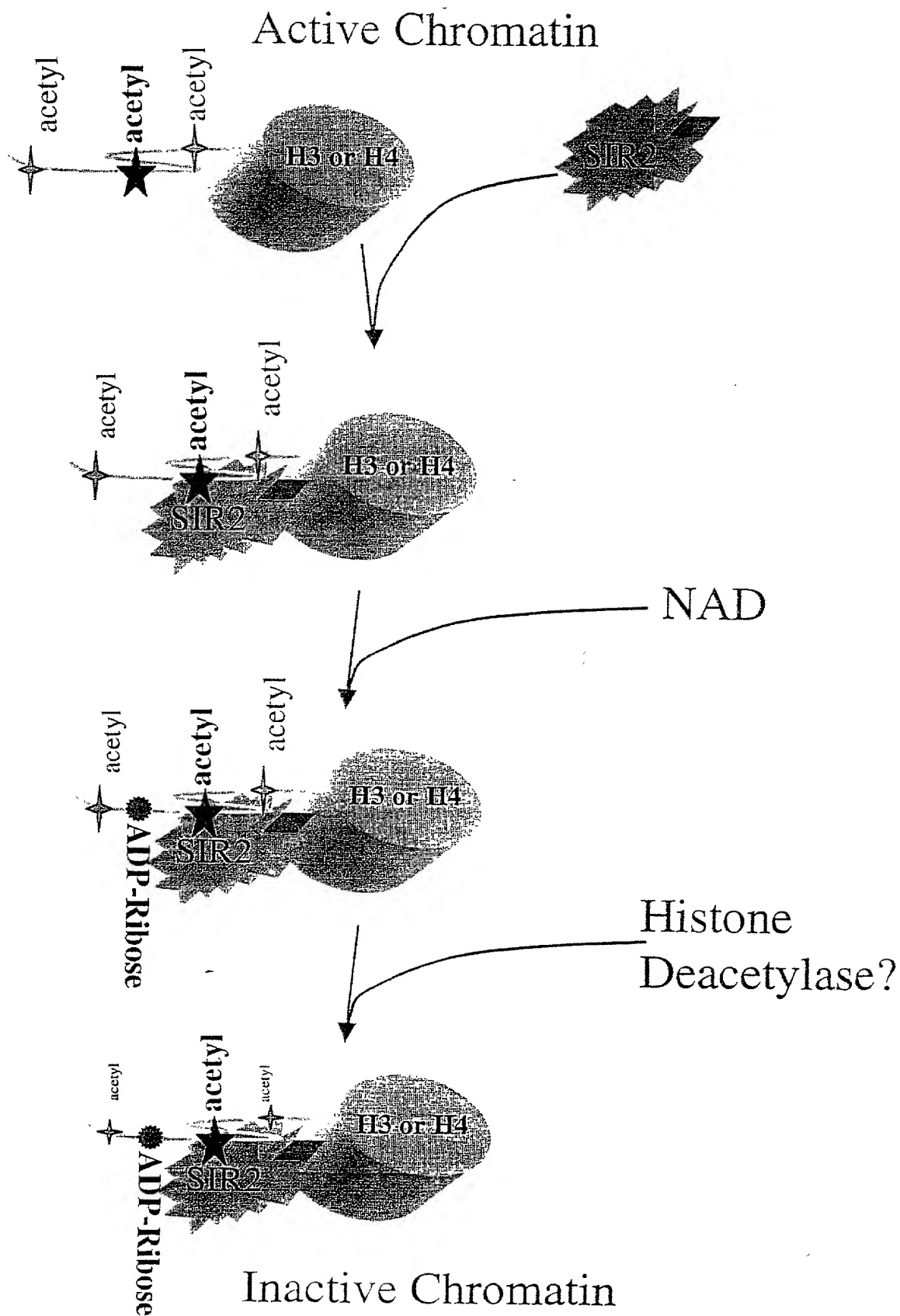


Figure 7



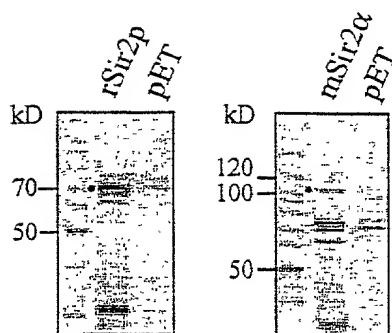


Figure 8a

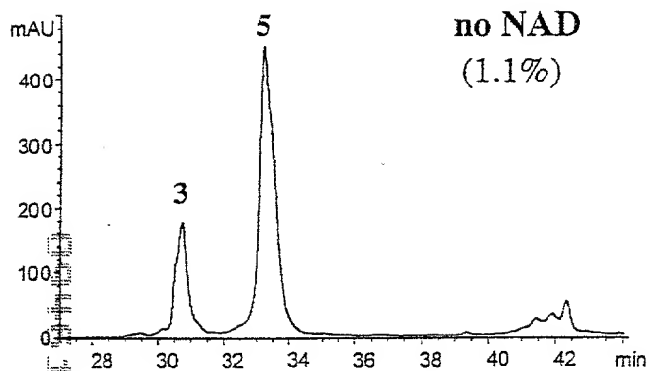


Figure 8b

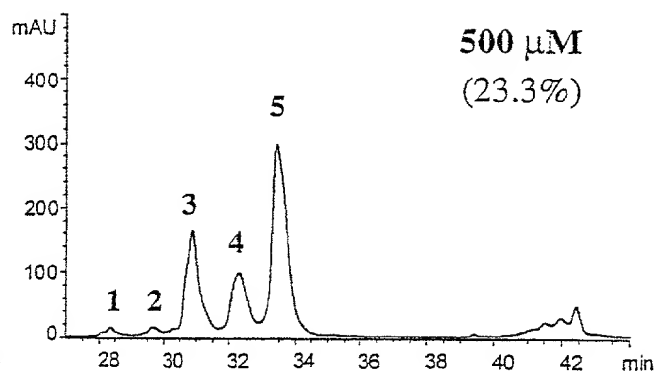


Figure 8e

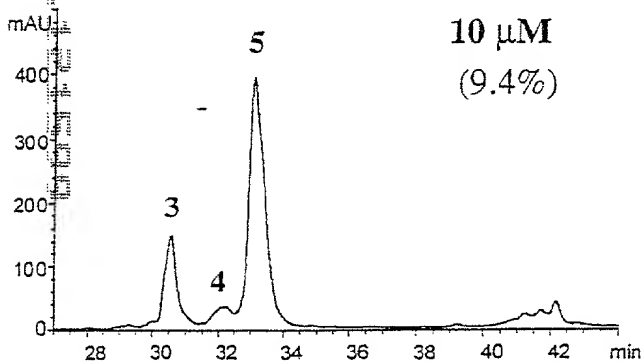


Figure 8c

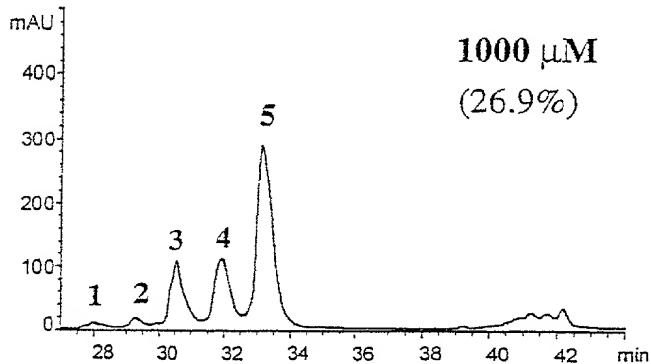


Figure 8f

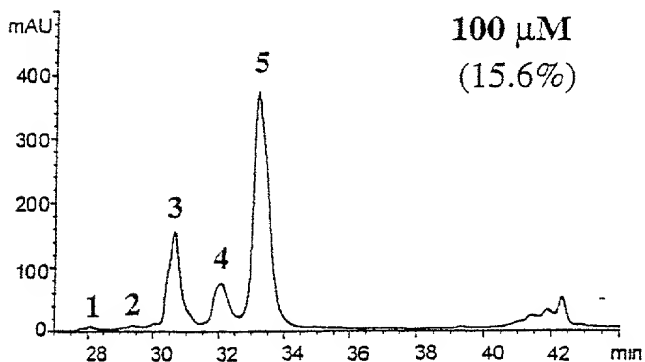


Figure 8d

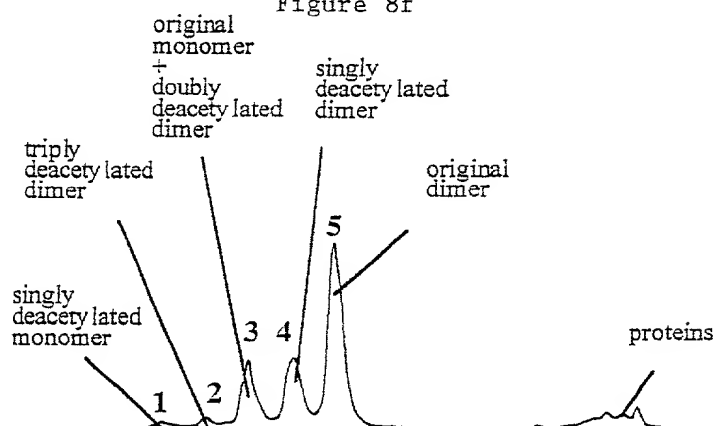


Figure 8g

Figure 9a

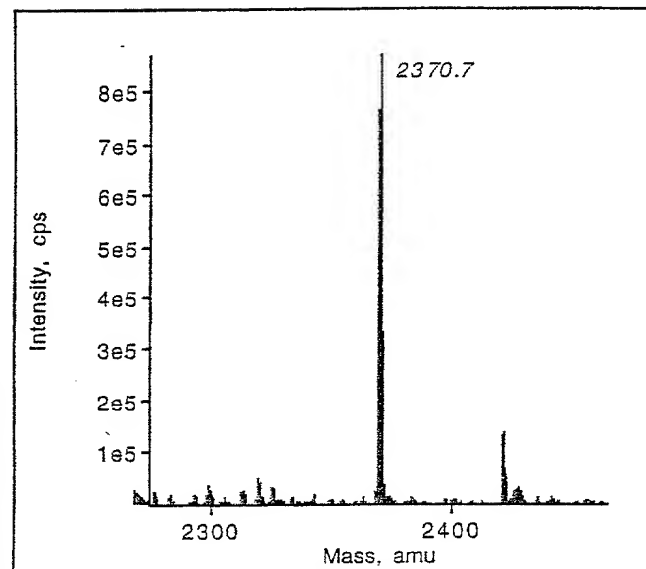


Figure 9b

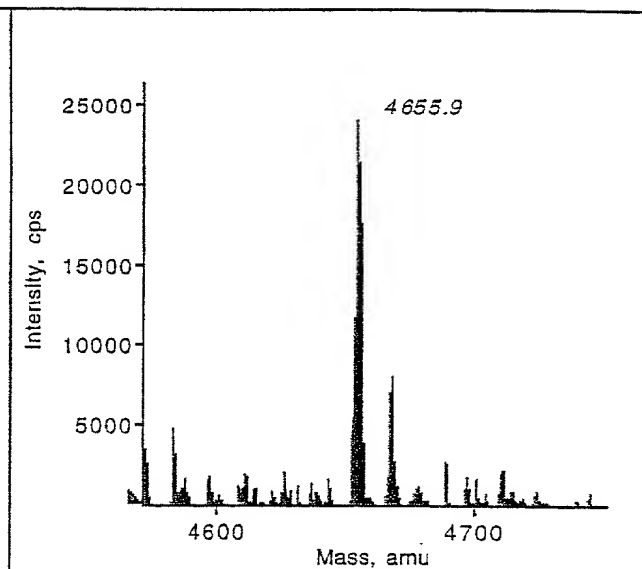


Figure 9c

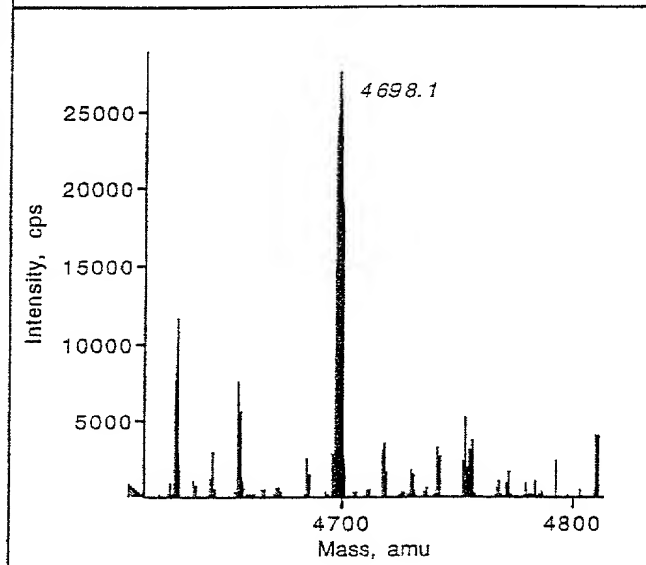
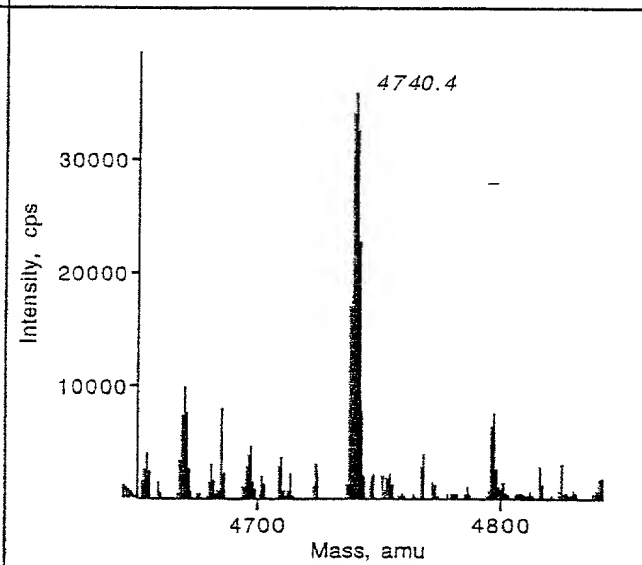


Figure 9d



peak 4

peak 5

9

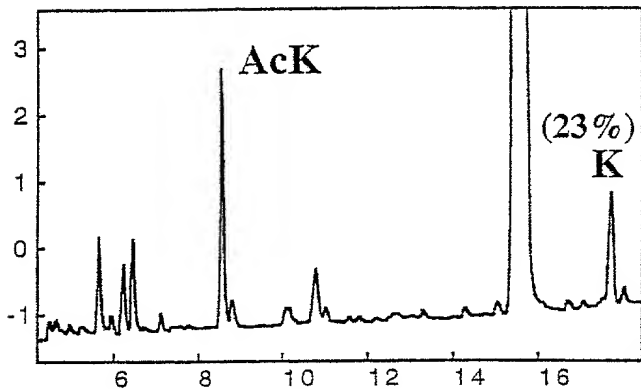


Figure 10a

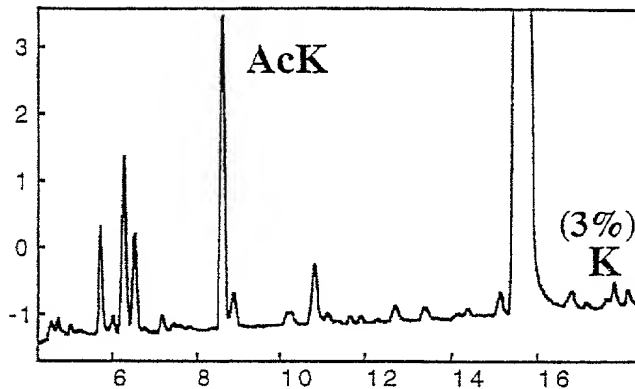


Figure 10d

14

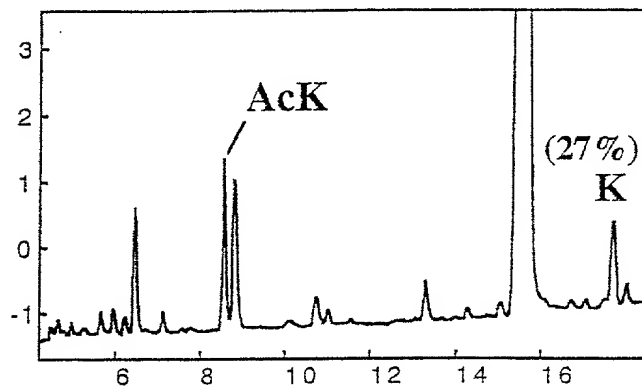


Figure 10b

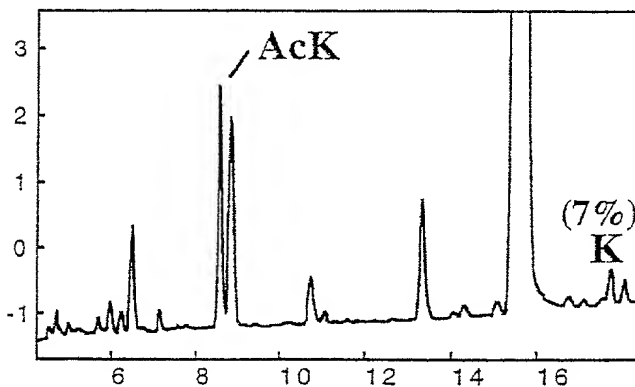


Figure 10e

18

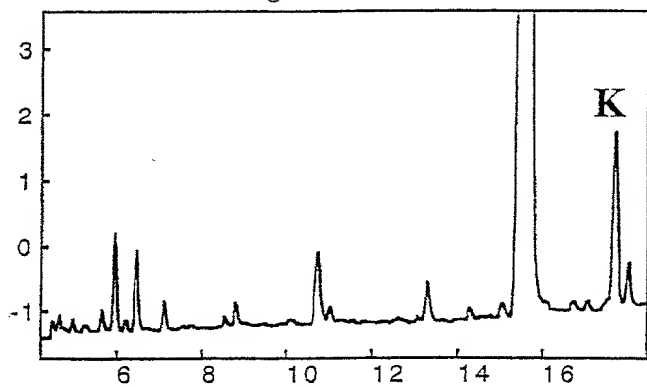


Figure 10c

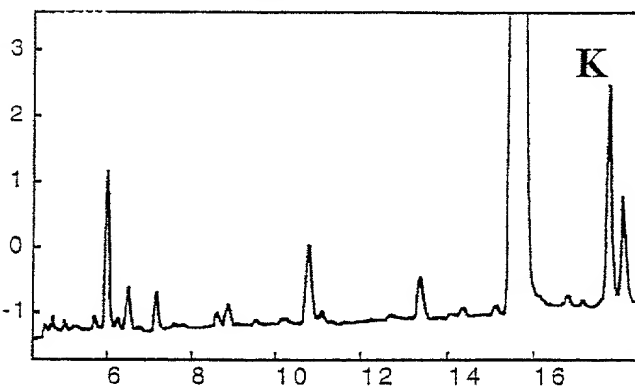


Figure 10f

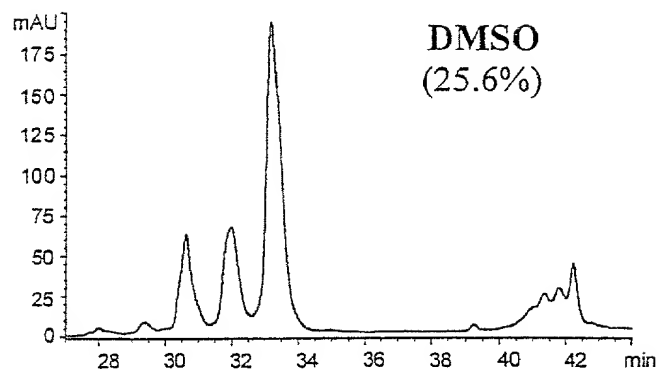


Figure 11a

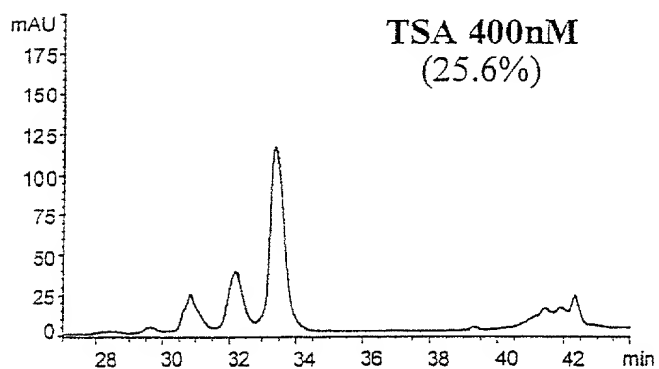


Figure 11b

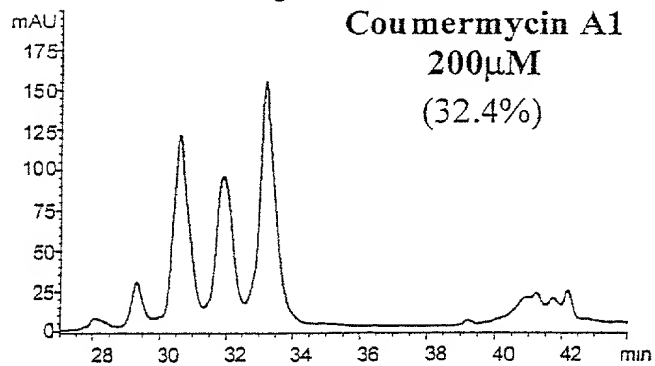


Figure 11e

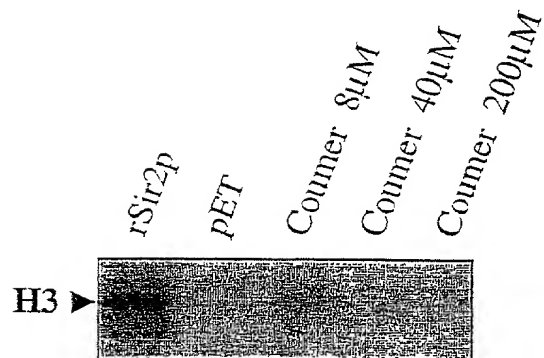


Figure 11c

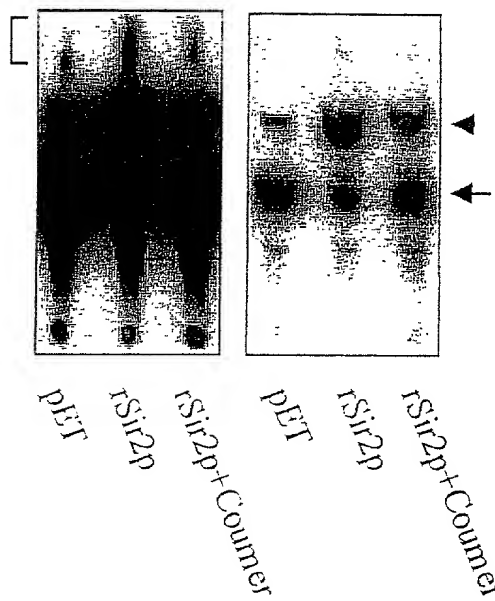


Figure 11d

1 MADEVA LA LQAA GSPS AAAAAA MEAA SQPADEP LKRP RRDG  
41 PGLGRSPGEP SA AVAPAAAGCEAA SAAAPAA LWREAAGAA  
81 ASAREAPAT AVAGDGN GSG LRHEPRAADD DDEGE EE  
121 DEAAAAA AAI GYRDNLL LT DGLLTNGFHSCE SDDDR T  
161 SHASSSDWTPRPRI GPYTFVQQHLMIGTDPRTI LKDLLPE  
201 TIPPPELDDMTLWQI VINI LSEPPKRRKKRDINTIEDAYK  
241 LLOECKI IIV LT GAGVSVSCG IPDFRSRDGI YARLAVDFP  
281 DLPDPQAMFDIEYFRKDP RPFKF AKEIYPGQFQPSLCHK  
321 FIALSDKEGK LLRNYTQNI DTLEOVAGIQRI LQCHGSFAI  
361 ASC LICKYKYDCEAVRGDIFNQVVP RCP RCPADEP LAIMK  
401 PEIVFFGENLPEQFHRAMKYDKDEV DLLIVIGSSLKVRPV  
441 ALIPSSIPHEVPQI LINREP LPHLHFDVE LLGDCDVIINE  
481 LCHRLGGEYAKLCCNPVKLSEITEKPPRQKELVH LSELP  
521 PTP LHI SEDSSPERTVPQDSSV IATLVDQATNNVNDLE  
561 VSESSCVEEK PQEVQTSRNVENI NVENPDFKAVGSSADK  
601 NERTSVAETVRKCPNRLAKEQI SKRLEGNOYLFVPPNRY  
641 IFHGAEVYSDSEDDVLS SSCGSNSDSGT CQSPS LEEPLE  
681 DESEIEEFYNGLEDDTERPECAGSGFGADGGDQEVVNEA  
721 IATRQELTDVNYPSDKS

Figure 12a

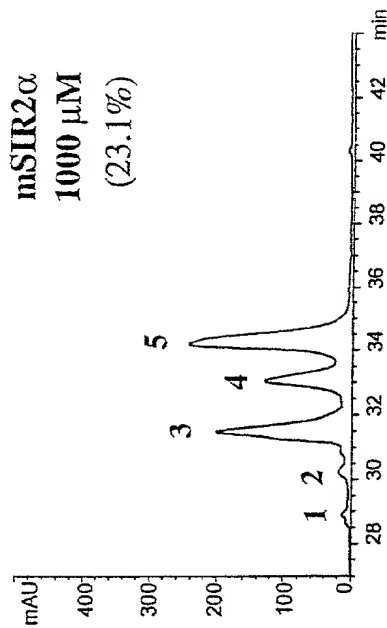


Figure 12c

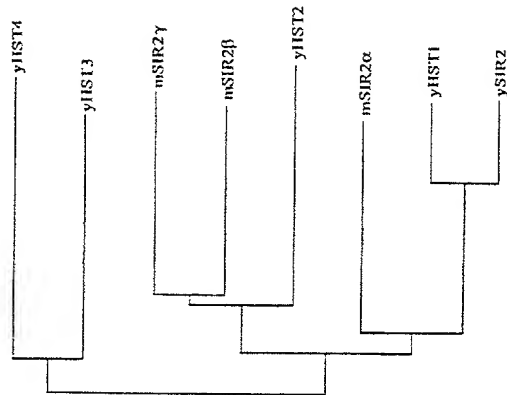


Figure 12b

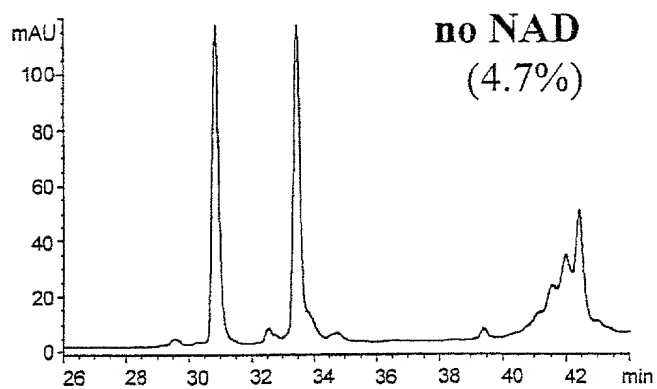


Figure 13a

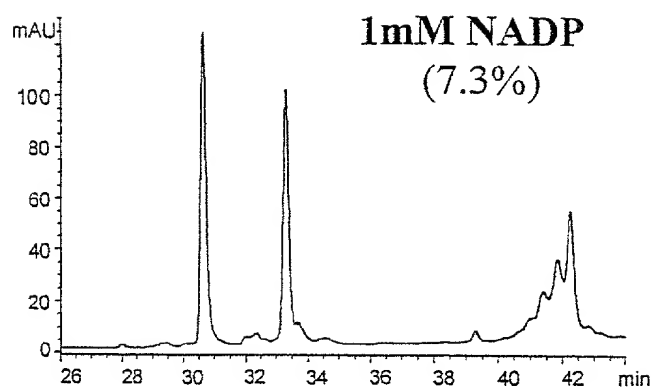


Figure 13d

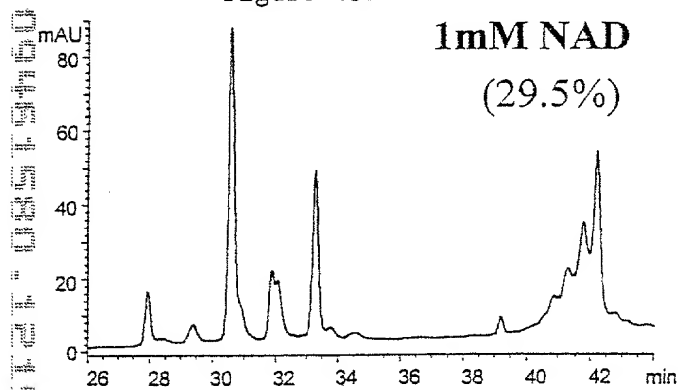


Figure 13b

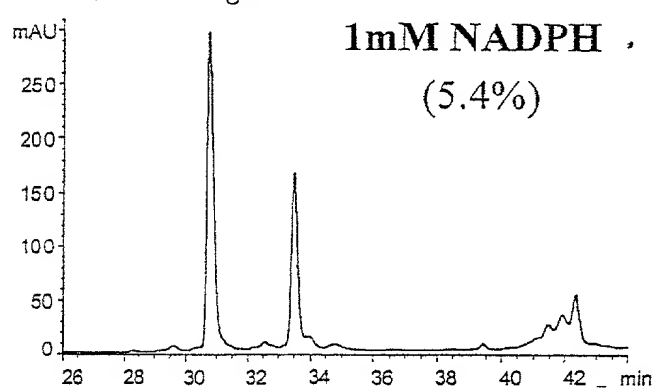


Figure 13e

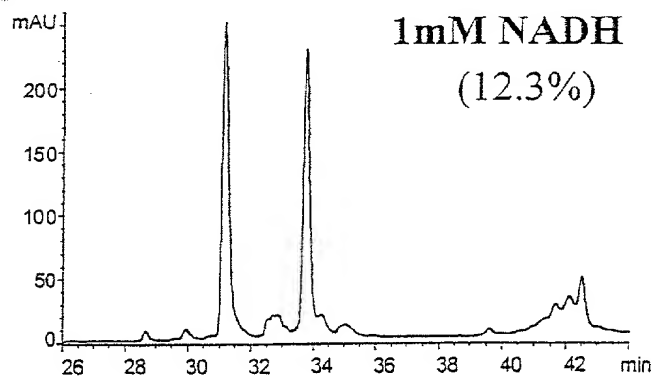


Figure 13c

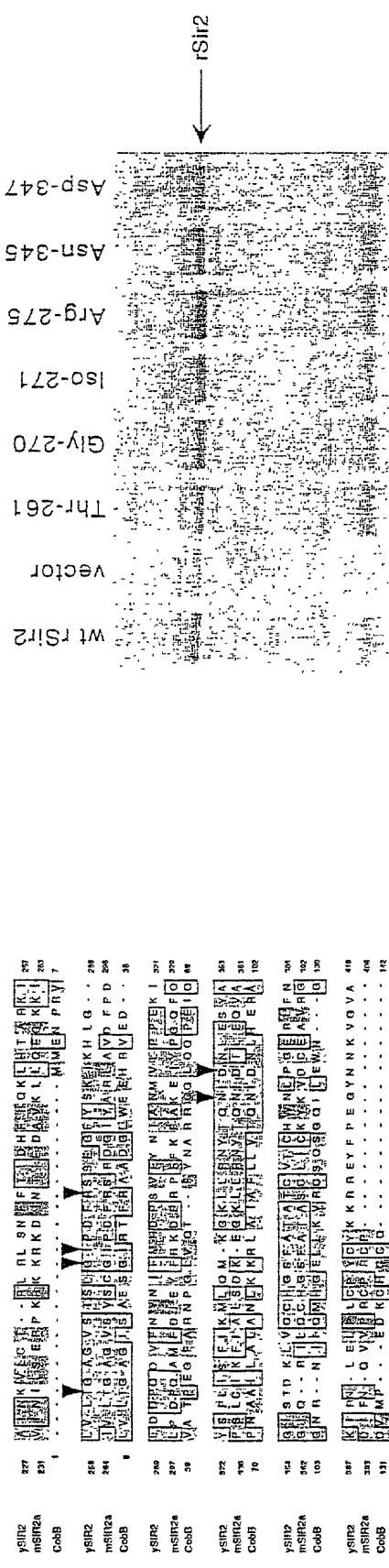


Figure 14b

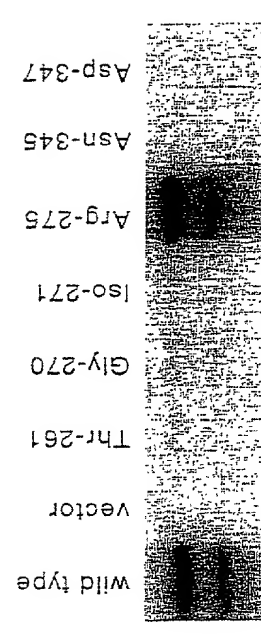


Figure 14c

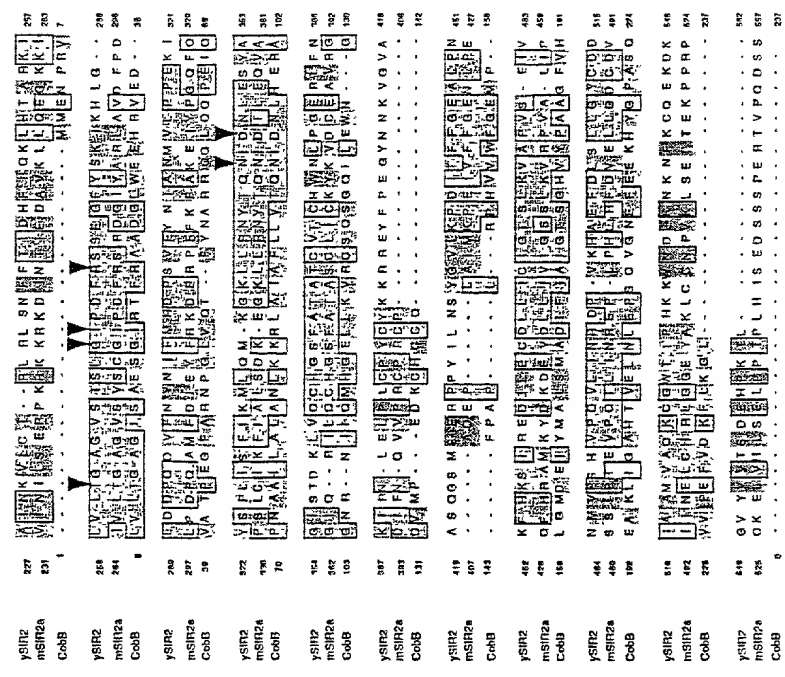


Figure 14a

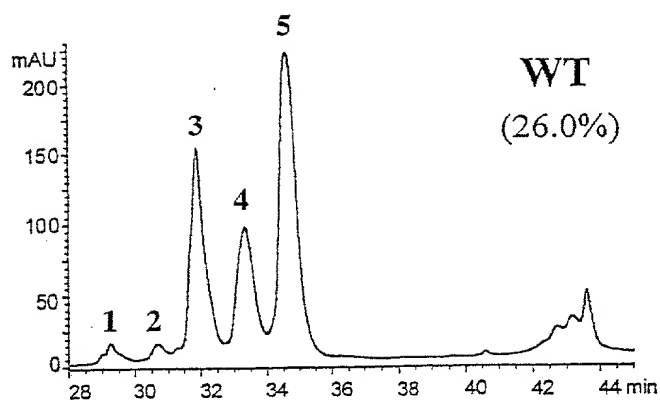


Figure 15a

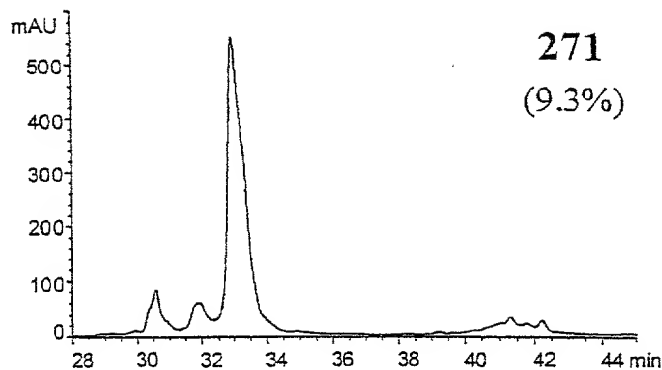


Figure 15e

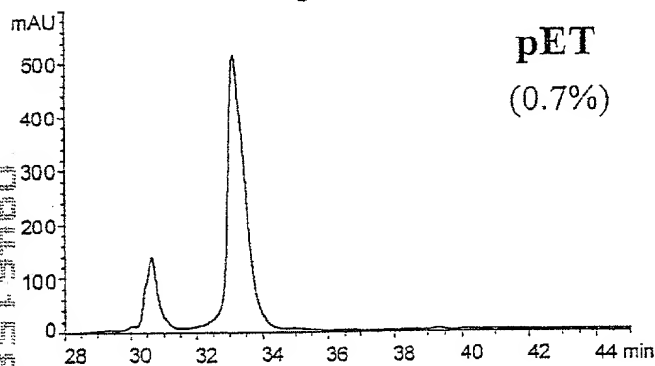


Figure 15b

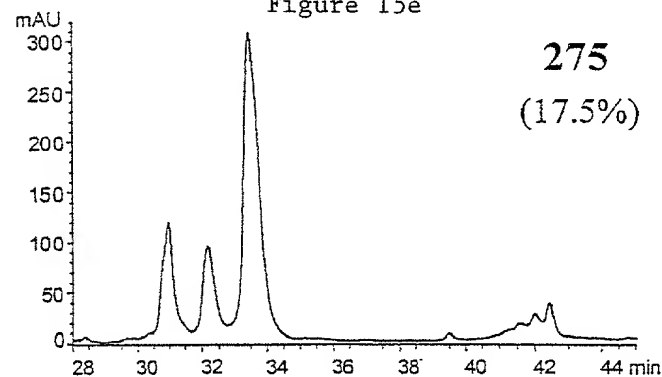


Figure 15f

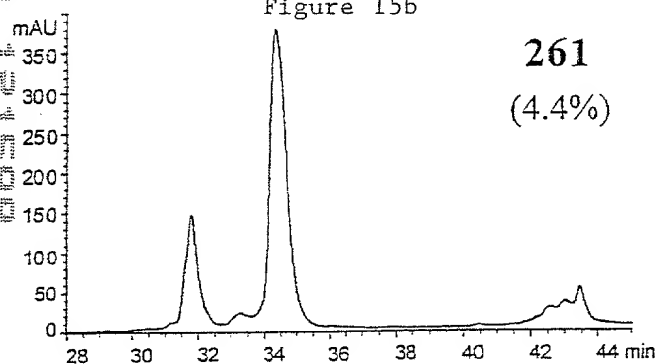


Figure 15c

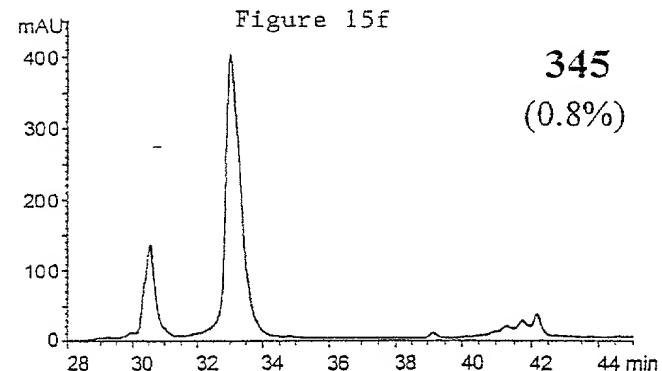


Figure 15g

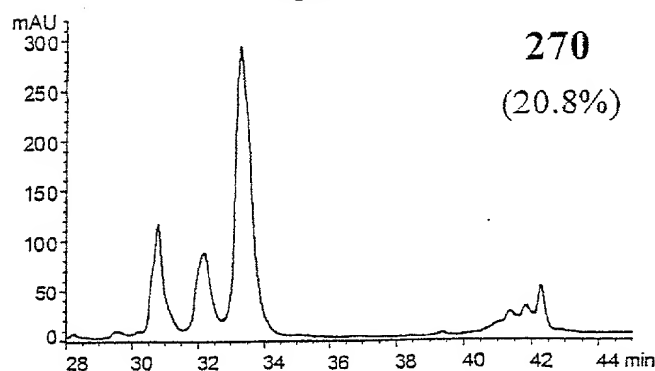


Figure 15d

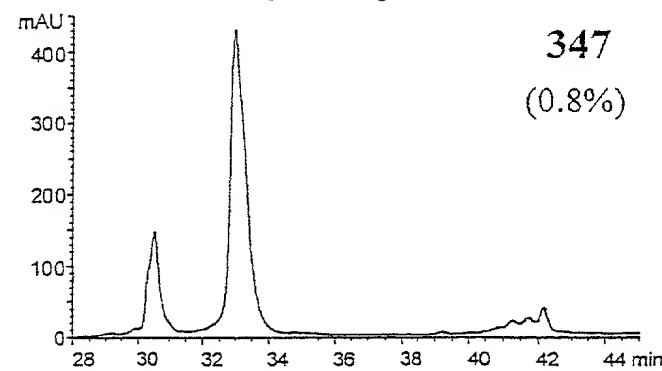


Figure 15h



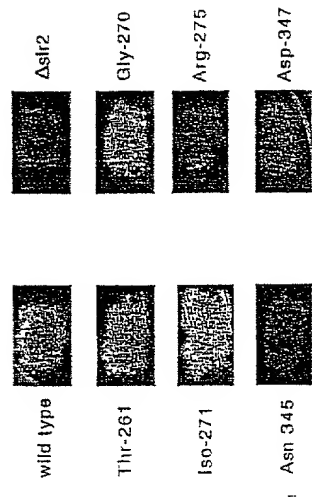


Figure 16b

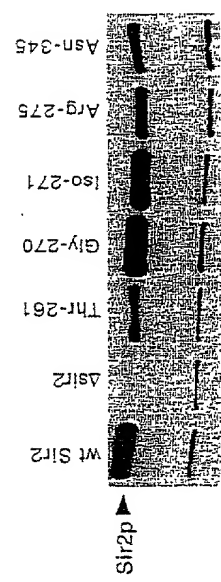


Figure 16a

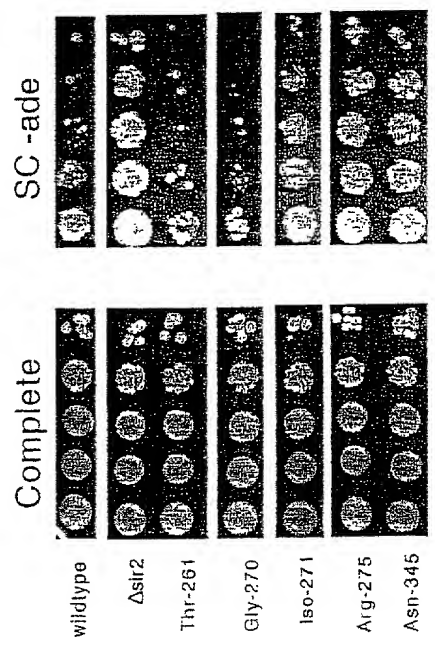


Figure 16c

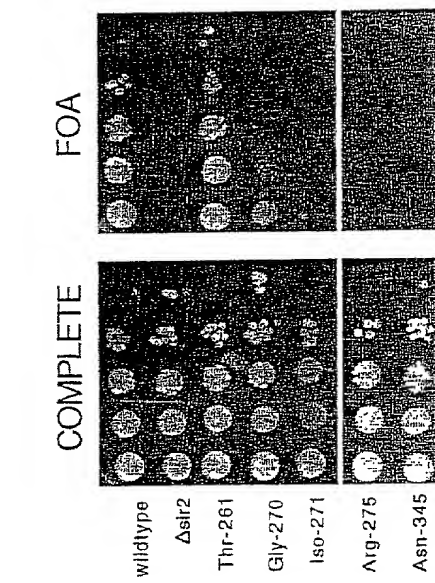


Figure 16d

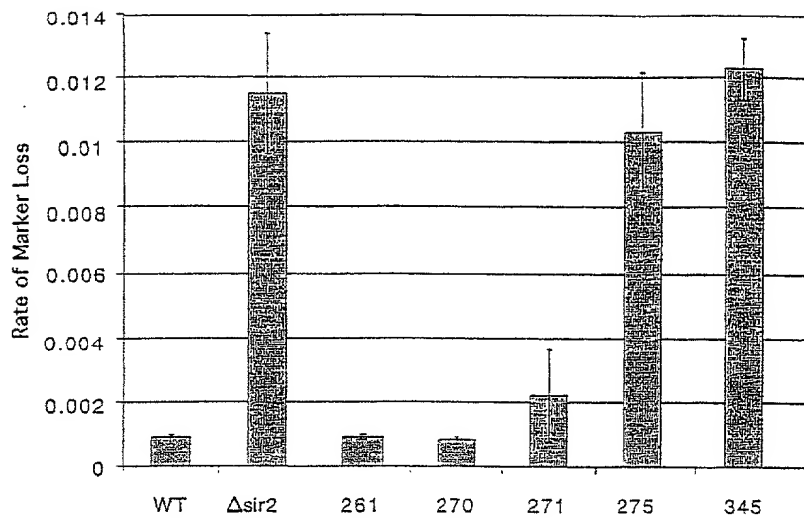


Figure 17a

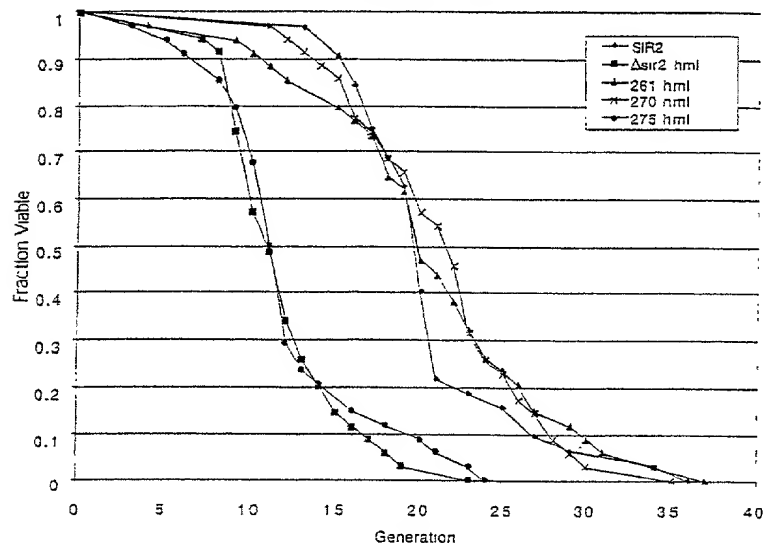


Figure 17b

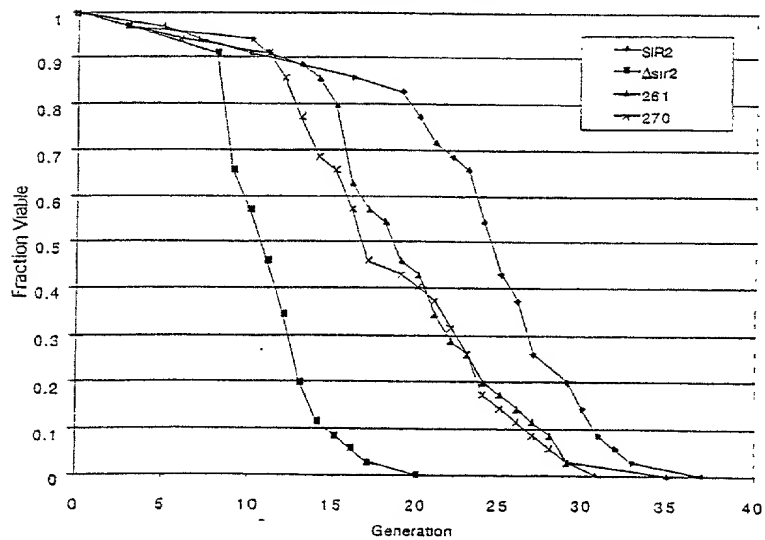


Figure 17c

Mutant	ADP-Rib. Activity (% of wt)	Deacetylase Activity (% of wt)	HM Silencing	Telomere Silencing	rDNA Silencing	rDNA Recombination	Mean Life Span (HML+)
<b>sir2Δ</b>	0%	2.7%	-	-	-	1.15%	11.4
<b>wildtype</b>	100%	100%	+	+	+	0.09%	24.4
<b>Thr-261</b>	4%	17%	+	+	+	0.09%	19.8
<b>Gly-270</b>	7%	80%	+	+/-	+	0.08%	18.9
<b>Iso-271</b>	8%	36%	+	-	+/-	0.22%	ND
<b>Arg-275</b>	100%	67%	-	-	-	1.03%	ND
<b>Asn-345</b>	0%	3%	-	-	-	1.22%	ND
<b>Asp-347</b>	0%	3%	-	ND	ND	ND	ND

Figure 18

ySIR2	257	I L V L T G A G V S T S L G I P D F R S - S E G F Y S K I K H - -	286
yHST1	203	I L V L T G A G V S T S L G I P D F R S - S E G F Y S K I R H - -	232
yHST2	27	V I F M V G A G I S T S G I P D F R S P G T G L Y H N L A R - -	57
yHST3	55	I A G L T G A G I S C N A G I P D F R S - S D G L Y D L V K K D C	86
yHST4	95	M V V V S G A G I S V A A G I P D F R S - S E G I F S T V N G G S	126
mSIR2alpha	263	I I V L T G A G V S V S G I P D F R S - R D G I Y A R L A V D F	294
mSIR2beta	79	V I G L V G A G I S T S A G I P D F R S P S T G L Y A N L E K - -	109
mSIR2g...	1	- -	10
AI465098	48	V V F H T G A G I S T A S G I P D F R G - P H G V W T M E E R - -	77
AI465820	67	L L V M T G A G I S T E S G I P D Y R S E K V G L Y A R T D R - -	97
AI466061	59	L A I S G A G V S A E S G V P T E R G - A G G Y W R K W Q A - -	88

ySIR2	287	- - - L G L D D P Q D V E N Y N I F M H D P S V - - F Y N I A N M	314
yHST1	233	- - - L G L E D P Q D V E N L D I F L Q D P S V - - F Y N I A H M	260
yHST2	58	- - - L K L P Y P E A M F D V D F F Q S D P L P - - F Y T L A K E	85
yHST3	87	S Q Y W S I K S G R E M E D I S L F R D D F K I S I F A K E M E R	119
yHST4	127	- - - G K D L F D Y N R V Y G D E S M S L K F N - - Q L M V S L F	154
mSIR2alpha	295	- - - P D L P D P Q A M E D I E Y F R K D P R P - - F F K F A K E	322
mSIR2beta	110	- - - Y H L P Y P E A I F E I S Y F K K H P E P - - F F A L A K E	137
mSIR2g...	11	- - - Y D P Y P E A I F E L G F F F N P K P - - F F M L A K E	38
AI465098	78	- - - G L A P K F D T I E E N A - - - - - - - - - - - - - - -	90
AI465820	98	- - - - - R P I Q - - H I D F V P V L R S A S G - - - - - - - -	114
AI466061	89	- - - Q D L A T P Q A F A R N P S Q V W E F Y H - - - - - Y R R E	113

ySIR2	315	V L P - - P E K I Y S P L H S P I K M L Q M K G K L L R N Y T O N	345
yHST1	261	V L P - - P E N M Y S P L H S F I K M L Q D K G K L L R N Y T O N	291
yHST2	86	L Y P - - G N F R P S K E H Y L L K L F Q D K D V L K R V Y T O N	116
yHST3	120	L Y S N V Q L A K P T K T H K F A H L K D R N K L L R C Y T O N	152
yHST4	155	R L S - - K N C Q R T K F H E M L N E F A R D G R L L R P L Y T O N	185
mSIR2alpha	323	L Y P - - G Q F Q P S L G H K F A L S D K E G K L L R N Y T O N	353
mSIR2beta	138	L Y P - - G Q F K P T I Q H Y F R L L K E K G L L L R C Y T O N	168
mSIR2g...	39	L Y P - - G H Y R R N V T H Y F L R L L D K E L L L R L Y T O N	69
AI465098	91	- - - - - R - - P S K T H M A L V O L E R M G F L S F L V S Q N	115
AI465820	115	T W P - - E N L W A G L N S P L T N P T Q H T W L - - - - - - -	137
AI466061	114	V M R - - S K - E P N P G H L A A Q C E A R - - - - - - - - -	133

ySIR2	346	I D N L E S Y A G I S T D - - - - - - - - - - - - - - - K L V Q	362
yHST1	292	I D N L E S Y A G I D P D - - - - - - - - - - - - - - - K L V Q	308
yHST2	117	I D T L E R Q A G V K D D - - - - - - - - - - - - - - - L I T E	133
yHST3	153	I D G L E E S I G L T L S N R K L P L T S F S S H W K N L D V V Q	185
yHST4	186	I D G L D T Q L P H L S T N - - - - - - - - - - - V P L A K P I P S T V Q	211
mSIR2alpha	354	I D T L E Q V A G I Q R - - - - - - - - - - - - - - - I L Q	368
mSIR2beta	169	I D T L E R V A G L E F Q - - - - - - - - - - - - - - - D L V E	185
mSIR2g...	70	I D G L E R A S G I P A S - - - - - - - - - - - - - - - K L V E	86
AI465098	116	V D G L D V R S G F P R D - - - - - - - - - - - - - - - K L A E	132
AI465820	0	- -	137
AI466061	0	- -	133

Figure 19

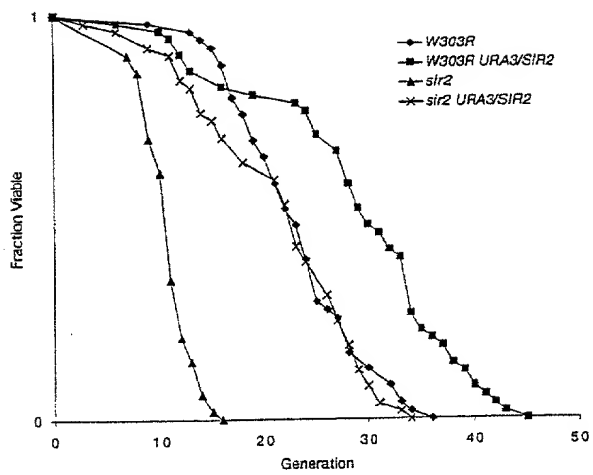


Figure 20

10 20 30 40 50 60  
 GCGGAGCAGAGGAGCGGAGGGCGGAGGGCCAGAGAGGCAGTTGGAAGATGGCGGACGAGG  
 M A D E V

70 80 90 100 110 120  
 TGGCGCTCGCCCTTCAGGCGCGCGGCTCCCCCTTCCGCGGCGGCGCCATGGAGGCGCGCT  
 A L A L Q A A G S P S A A A A M E A A S

130 140 150 160 170 180  
 CGCAGCCCGGCGGACGAGCCGCTCCGCAAGAGGCCCCGCGGAGACGGGCGCTGGCCTCGGGC  
 Q P A D E P L R K R P R R D G P G L G R

190 200 210 220 230 240  
 GCAGCCCCGGGCGAGCCGAGCGCAGCAGTGGCGCCGCGCGCGCGGGGTGTGAGGCGGCGA  
 S P G E P S A A V A P A A A G C E A A S

250 260 270 280 290 300  
 GCGCCGCGGCCCCGGCGGCGCTGTGGCGGGGAGCGCGCAGGGGCGGCGGCGAGCGCGGAGC  
 A A A P A A L W R E A A G A A A S A E R

310 320 330 340 350 360  
 GCGAGGCCCCGGGCGACGGCCGTGGCCGGGGACGGAGACAATGGGTCCGGCCTGCGGCGGG  
 E A P A T A V A G D G D N G S G L R R E

370 380 390 400 410 420  
 AGCCGAGGGCGGCTGACGACTTCGACGACGACGAGGGCGAGGAGGAGGACGAGGCGGCGG  
 P R A A D D F D D D E G E E E D E A A A

430 440 450 460 470 480  
 CGGCAGCGGCGGCGGCGGCGATCGGCTACCGAGACAACCTCCTGTTGACCGATGGACTCC  
 A A A A A A I G Y R D N L L L T D G L L

490 500 510 520 530 540  
 TCACTAATGGCTTTCATTCCCTGTGAAAGTGATGACGATGACAGAACGTCACACGCCAGCT  
 T N G F H S C E S D D D D R T S H A S S

550 560 570 580 590 600  
 CTAGTGACTGGACTCCGCGGCGCGGATAGGTCCATATACTTTTGTTCAGCAACATCTCA  
 S D W T P R P R I G P Y T F V Q Q H L M

610 620 630 640 650 660  
 TGATTGGCACCGATCCTCGAACAATTCTTAAAGATTTATTACCAGAAACAATTCCTCCAC  
 I G T D P R T I L K D L L P E T I P P P

670 680 690 700 710 720  
 CTGAGCTGGATGATATGACGCTGTGGCAGATTGTTATTAAATATCCTTTTCAGAACCAACAA  
 E L D D M T L W Q I V I N I L S E P P K

730 740 750 760 770 780  
 AGCGGAAAAAAGAAAAAGATATCAATACAATTGAAGATGCTGTGAAGTTACTGCAGGAGT  
 R K K R K D I N T I E D A V K L L Q E C

790 800 810 820 830 840  
 GTAAAAACATAATAGTTCTGACTGGAGCTGGGGTTTCTGTCTCCTGTGGGATTCTGACT  
 K K I I V L T G A G V S V S C G I P D F

850 860 870 880 890 900  
 TCAGATCAAGAGACGGTATCTATGCTCGCCTTGCGGTGGACTTCCCAGACCTCCCAGACC  
 R S R D G I Y A R L A V D F P D L P D P

Figure 21a

910 920 930 940 950 960  
 CTC AAGCCATGTTT GATATTG AGTATTT TAGAAAAGACCC AAGACCATTCCTTCAAGTTTG  
 Q A M F D I E Y F R K D P R P F F K F A

970 980 990 1000 1010 1020  
 CAAAGGAAATATATCCCGGACAGTTCCAGCCGTCTCTGTGTGCACAAATTCATAGCTTTGT  
 K E I Y P G Q F Q P S L C H K F I A L S

1030 1040 1050 1060 1070 1080  
 CAGATAAGGAAGGAAA ACTACTTCGAAAT TATACTCAAAATATAGATACCTTGGAGCAGG  
 D K E G K L L R N Y T Q N I D T L E Q V

1090 1100 1110 1120 1130 1140  
 TTGCAGGAATCCAAAGGATCCTTCAGTGT CATGGTTCCTTTGCAACAGCATCTTGCCTGA  
 A G I Q R I L Q C H G S F A T A S C L I

1150 1160 1170 1180 1190 1200  
 TTTGTAAATACAAAGTTGATTGTGAAGCTGTTTCGTGGAGACATTTTAAATCAGGTAGTTC  
 C K Y K V D C E A V R G D I F N Q V V P

1210 1220 1230 1240 1250 1260  
 CTCGGTGCCCTAGGTGCCCAGCTGATGAGCCACTTGCCATCATGAAGCCAGAGATTGTCT  
 R C P R C P A D E P L A I M K P E I V F

1270 1280 1290 1300 1310 1320  
 TCTTTGGTGAAAACCTTACCAGAACAGTTTCATAGAGCCATGAAGTATGACAAAGATGAAG  
 F G E N L P E Q F H R A M K Y D K D E V

1330 1340 1350 1360 1370 1380  
 TTGACCTCCTCATTTGTTATTGGATCTTCTCTGAAAGTGAGACCAGTAGCACTAATTCCAA  
 D L L I V I G S S L K V R P V A L I P S

1390 1400 1410 1420 1430 1440  
 GTTCTATACCCCATGAAGTGCCTCAAATATTAATAAATAGGGAACCTTTGCCTCATCTAC  
 S I P H E V P Q I L I N R E P L P H L H

1450 1460 1470 1480 1490 1500  
 ATTTTGTAGTGTAGAGCTCCTTGGAGACTGCGATGTTATAATTAATGAGTTGTGTGCATAGGC  
 F D V E L L G D C D V I I N E L C H R L

1510 1520 1530 1540 1550 1560  
 TAGGTGGTGAATATGCCAAACCTTTGTTGTAACCCCTGTAAAGCTTTTCAGAAATTAAGTGA  
 G G E Y A K L C C N P V K L S E I T E K

1570 1580 1590 1600 1610 1620  
 AACCTCCACGCCACAAAAGGAATTGGTTTCATTTATCAGAGTTGCCACCAACACCTCTTC  
 P P R P Q K E L V H L S E L P P T P L H

1630 1640 1650 1660 1670 1680  
 ATATTTCCGGAAGACTCAAGTTTCACCTGAAAGAACTGTACCACAAGACTCTTCTGTGATTG  
 I S E D S S S P E R T V P Q D S S V I A

1690 1700 1710 1720 1730 1740  
 CTACACTTGTAGACCAAGCAACAAACAACAAATGTTAATGATTTAGAAGTATCTGAATCAA  
 T L V D Q A T N N N V N D L E V S E S S

1750 1760 1770 1780 1790 1800  
 GTTGTGTGGAAGAAAAACCACAAGAAGTACAGACTAGTAGGAATGTTGAGAACATTAATG  
 C V E E K P Q E V Q T S R N V E N I N V

1810 1820 1830 1840 1850 1860  
 TGGAAAATCCAGATTTTAAAGGCTGTTGGTTCCAGTACTGCAGACAAAAATGAAAGAACTT  
 E N P D F K A V G S S T A D K N E R T S

1870 1880 1890 1900 1910 1920  
 CAGTTGCAGAAACAGTGAGAAAATGCTGGCCTAATAGACTTGCAAAGGAGCAGATTAGTA  
 V A E T V R K C W P N R L A K E Q I S K

Figure 21b

1930 1940 1950 1960 1970 1980  
 AGCGGCTTGAGGGTAATCAATACCTGTTTTGTACCACCAAATCGTTACATATTCCACGGTG  
 R L E G N Q Y L F V P P N R Y I F H G A

1990 2000 2010 2020 2030 2040  
 CTGAGGTATACTCAGACTCTGAAGATGACGTCTTGTCTCTAGTTCTGTGGCAGTAACA  
 E V Y S D S E D D V L S S S S C G S N S

2050 2060 2070 2080 2090 2100  
 GTGACAGTGGCACATGCCAGAGTCCAAGTTTAGAAGAACCCTTGGAAGATGAAAGTGAAA  
 D S G T C Q S P S L E E P L E D E S E I

2110 2120 2130 2140 2150 2160  
 TTGAAGAATTCTACAATGGCTTGGAAGATGATACGGAGAGGCCCGAATGTGCTGGAGGAT  
 E E F Y N G L E D D T E R P E C A G G S

2170 2180 2190 2200 2210 2220  
 CTGGATTGAGCTGATGGAGGGGATCAAGAGGTTGTTAATGAAGCTATAGCTACAAGAC  
 G F G A D G G D Q E V V N E A I A T R Q

2230 2240 2250 2260 2270 2280  
 AGGAATTGACAGATGTAAACTATCCATCAGACAAATCATAACACTATTGAAGCTGTCCGG  
 E L T D V N Y P S D K S \*

2290 2300 2310 2320 2330 2340  
 AATCAGGAATTGCTCCACCAGCATTTGGGAACCTTAGCATGTCAAAAAATGAATGTTTAC

2350 2360 2370 2380 2390 2400  
 TTGTGAACCTTGAACAAGGAAATCTGAAAGATGTATTATTTATAGACTGGAAAATAGATTG

2410 2420 2430 2440 2450 2460  
 TCTTCTTGGATAATTTCTAAAGTTCCATCATTTCTGTTTGTACTTGTACATTCAACACTG

2470 2480 2490 2500 2510 2520  
 TTGGTTGACTTCATCTTCTTCAAGGTTTCTTGTATGATACATTCTGTATGTATGTATA

2530 2540 2550 2560 2570 2580  
 AATTTGTTTTTTTGCCTAATGAGTTTCAACCTTTTAAAGTTTTTCAAAGCCATTGGAATGT

2590 2600 2610 2620 2630 2640  
 TAATGTAAAGGGAACAGCTTATCTAGACCAAAGAATGGTATTTTACACTTTTTTGTGTGT

2650 2660 2670 2680 2690 2700  
 AACATTGAATAGTTTAAAGCCCTCAATTTCTGTTCTGCTGAACTTTTATTTTTAGGACAG

2710 2720 2730 2740 2750 2760  
 TTAACTTTTTAAACACTGGCATTTTTCCAAACTTGTGGCAGCTAACTTTTTTAAATCACA

2770 2780 2790 2800 2810 2820  
 GATGACTTGTAAATGTGAGGAGTCAGCACCGTGTCTGGAGCACTCAAACTTGGGCTCAGT

2830 2840 2850 2860 2870 2880  
 GTGTGAAGCGTACTTACTGCATCGTTTTTGTACTTGTCTGCAGACGTGGTAATGTCCAAAC

2890 2900 2910 2920 2930 2940  
 AGGCCCTGAGACTAATCTGATAAATGATTTGGAAATGTGTTTTCAGTTGTTCTAGAAACA

2950 2960 2970 2980 2990 3000  
 ATAGTGCCCTGTCTATATAGGTCCCTTAGTTTGAATATTTGCCATTGTTTAAATTAAATAC

3010 3020 3030 3040 3050 3060  
 CTATCACTGTGGTAGAGCCTGCATAGATCTTCAACCACAAATACTGCCAAGATGTGAATAT

3070 3080 3090 3100 3110 3120  
 GCAAAGCCTTTTCTGAATCTAATAATGGTACTTCTACTGGGGAGAGTGAATATTTTGGAC

3130 3140 3150 3160 3170 3180  
 TGCTGTTTTTCCATTAAATGAGGAAAGCAATAGGCCTCTTAATTAAAGTCCCAAAGTCATA

Figure 21c



3190 3200 3210 3220 3230 3240  
 AGATAAAATTGTAGCTCAACCAGAAAGTACACTGTTGCCTGTTGAGGATTTGGTGTAAATGT  
 3250 3260 3270 3280 3290 3300  
 ATCCCAAGGTGTTAGCCTTGTATTATGGAGATGAATACAGATCCAATAGTCAAATGAAAC  
 3310 3320 3330 3340 3350 3360  
 TAGTTCCTTAGTTATTTAAAAGCTTAGCTTGCCTTAAAAC TAGGGATCAATTTTCTCAACT  
 3370 3380 3390 3400 3410 3420  
 GCAGAAACTTTTAGCCTTTCAAACAGTTCACACCTCAGAAAGTCAGTATTTATTTTACAG  
 3430 3440 3450 3460 3470 3480  
 ACTTCTTTGGAACATTGCCCCCAAATTTAAATATTCATGTGGGTTTAGTATTTATTACAA  
 3490 3500 3510 3520 3530 3540  
 AAAAATGATTTGAAATATAGCTGTTCTTTATGCATAAAATACCCAGTTAGGACCATTACT  
 3550 3560 3570 3580 3590 3600  
 GCCAGAGGAGAAAAGTATTAAGTAGCTCATTTCCCTACCTAAAAGATAACTGAATTTATT  
 3610 3620 3630 3640 3650 3660  
 TGGCTACACTAAAGAATGCAGTATATTTAGTTTTCCATTTGCATGATGTGTTTGTGCTAT  
 3670 3680 3690 3700 3710 3720  
 AGACAATATTTTAAATTGAAAAATTTGTTTTAAATTATTTTTACAGTGAAGACTGTTTTTC  
 3730 3740 3750 3760 3770 3780  
 AGCTCTTTTTATATTGTACATAGACTTTTATGTAATCTGGCATATGTTTTGTAGACCGTT  
 3790 3800 3810 3820 3830 3840  
 TAAATGACTGGATTATCTTCCTCCAAC TTTTGAAATACAAAAACAGTGTTTTATACTAAAA  
 3850 3860 3870  
 AAAAAAAAAAGTCGACGCGGCCGCGAATTC

Figure 2ld

10 20 30 40 50 60  
 CCACGCGTCCGCGGACGCGTGGGCACGGGACAGAGCAGTCGGTGACAGTCCCGAGGGCCC  
 T R P R T R G H G T E Q S V T V P R A P

70 80 90 100 110 120  
 CCACCCCGTTCCCATGGCCGAGCCGGACCGATTTCAGACTCGGACACTGAGGGAGGAGCCA  
 T P F P W P S R T D S D S D T E G G A T

130 140 150 160 170 180  
 CTGGTGAGAGGCAGAGATGGACTTCCTGAGGAATTTATTACCCAGACCCTGGGCCTGG  
 G G E A E M D F L R N L F T Q T L G L G

190 200 210 220 230 240  
 GTCCCAAAGGAGCGTCTTCTAGACGAGCTGACCCTCGAAGGAGTGACACGCTACATGC  
 S Q K E R L L D E L T L E G V T R Y M Q

250 260 270 280 290 300  
 AGAGCGAGCGCTGCCGCAAGGTCATCTGTTTGGTGAGCCGGAATCTCCACGTCGCGGG  
 S E R C R K V I C L V G A G I S T S A G

310 320 330 340 350 360  
 GATCCCTGACTTCCGCTCCCCGTCCACTGGCCTCTATGCAAACCTGGAGAAGTACCACC  
 I P D F R S P S T G L Y A N L E K Y H L

370 380 390 400 410 420  
 TTCCTTACCCAGAGGCCATCTTTGAGATCAGCTACTTCAAGAAACATCCGGAACCCTTCT  
 P Y P E A I F E I S Y F K K H P E P F F

430 440 450 460 470 480  
 TTGCCCTTGCCAAGGAGCTCTATCCCGGGCAGTTCAAGCCAACCATCTGCCACTACTTCA  
 A L A K E L Y P G Q F K P T I C H Y F I

490 500 510 520 530 540  
 TCCGCCTGCTGAAGGAGAAGGGGCTGCTGCTGCGCTGCTACACGCAGAACATAGACACGC  
 R L L K E K G L L L R C Y T Q N I D T L

550 560 570 580 590 600  
 TGGAACGAGTGGCGGGGCTGGAGCCCCAGGACCTGGTGGAGGCCACGGCACCTTCTACA  
 E R V A G L E P Q D L V E A H G T F Y T

610 620 630 640 650 660  
 CATCACACTGTGTCAACACCTCCTGCAGAAAAGAATACACGATGGGCTGGATGAAAGAGA  
 S H C V N T S C R K E Y T M G W M K E K

670 680 690 700  
 AGATTTCCTCAGAAGCAACTCCCAGGTGTGAGCAGTGTCA  
 I S Q K Q L P G V S S V

Figure 22